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The effects of ageing on cutaneous immunity

Submitted by
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I, Katie Lacy confirm that the work presented in this thesis is my own. Where information has been derived from other sources I confirm that this has been indicated in the thesis.

Signed:

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Dedication

I would like to dedicate this thesis to my father, Professor John Birch, for his life-long support and who was influential in encouraging me to undertake a PhD.

Abstract

This work investigated the effects of ageing on the cutaneous response to the injection of bacterial, fungal and viral antigens in humans. Previous studies have shown reduced cutaneous delayed type hypersensitivity (DTH) responses in the old and it has been assumed that this is due to an overall decline in cell- mediated immunity that occurs with ageing. However, it has remained unclear as to whether DTH responses in old skin are truly reflective of immune responses in all compartments of the immune system. Reduced clinical responsiveness in the skin to the intradermal injection of antigen was identified despite good peripheral T cell responses in the old and it was hypothesised that this may be reflective of a skin-specific defect that occurs during ageing. Possible mechanisms for this were investigated by performing both skin suction blisters and skin biopsies following injection of antigen into the skin. In the old there was a significant reduction in the infiltration of CD4+ lymphocytes in the skin that correlated with a reduced clinical response. A reduction in CXCR3 expression was found on CLA+CD4+ T cells isolated from the peripheral blood in old subjects. In addition, a reduction in the levels of cutaneous pro- inflammatory mediators was identified. This was associated with reduced CD4+ T cell activation and proliferation *in situ* as determined by CD69 and Ki67 expression on lymphocytes isolated directly *ex vivo* from the skin. A defect in initiation of the DTH response was considered. Although a possible decrease in the numbers of CD14+ monocytes was identified, no difference in monocyte activation or the numbers of CD1a or DC-SIGN positive dendritic cells was found in the old. The data suggests that both decreased cell migration in to the skin in addition to decreased expansion of infiltrating cells contributes to the defective cutaneous response to antigenic challenge during ageing.

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Abbreviations

APC	Antigen presenting cell
APC	Allophycocyanin
BCG	Bacillus of Calmette and Guérin
BSA	Bovine serum albumin
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	Cluster of differentiation
CLA	Cutaneous lymphocyte antigen
CMV	Cytomegalovirus
CNS	Central nervous system
Con A	Concanavalin A
Cpm	Counts per minute
CTACK	Cutaneous T cell-attracting chemokine
Cy5	Cyanin 5
DC	Dendritic cell
DDC	Dermal dendritic cell
DMEC	Dermal microvascular endothelial cell
DTH	Delayed type hypersensitivity
DKC	Dyskeratosis congenita
EBV	Epstein Barr Virus
EI	Erythema-index
E-selectin	Endothelial cell selectin
FCS	Foetal calf serum
FITC	Fluoroscein isothiocyanate
FSC	Forward scatter
GM-CSF	Granulocyte monocyte-colony stimulating factor
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILT3	Immunoglobulin like transcript 3

IP-10	Interferon gamma inducible protein 10
I-TAC	Interferon T cell alpha chemoattractant
JAK	Janus kinase
HLA	Human leukocyte antigen
KC	Keratinocyte
LC	Langerhans cell
LFA	Leucocyte function-associated antigen
LPS	Lipopolysaccharide
MDC	Macrophage derived chemokine
MFI	Mean fluorescent intensity
MHC	Major histocompatibility complex
Mig	Monokine induced by interferon gamma
MIP	Macrophage-inflammatory protein
MT	Mantoux test
mRNA	Messenger ribonucleic acid
NFAT	Nuclear factor of activated T cells
PAMPs	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBSA	Phosphate buffered saline with bovine serum albumin and sodium azide
PDC	Plasmacytoid dendritic cell
PE	Phycoerythrin
PECy5.5	Phycoerythrin-cyanin 5.5
PerCP	Peridinin chlorophyll protein
PHA	Phytohemagglutinin
PKC	Protein kinase C
PPD	Tuberculin purified protein derivative
P-selectin	Platelet selectin
PRRs	Pattern recognition receptors
RBC	Red blood cell
RPMI	Roselyn Park Memorial Institute
SB	Skin suction blister
SD	Standard deviation

SDF	Stromal cell-derived factor
SEB	Staphylococcal enterotoxin B
SEM	Standard error of the mean
SLC	Secondary lymphoid chemokine
SSC	Side scatter
SCID	Severe combined immune deficiency
STAT	Signal tranducers and activators of transcription
T_{CM}	Central memory T cell
T_{EM}	Effector memory T cell
T_H	T helper cell
T_{reg}	Regulatory T cells
TARC	Thymus- and activation regulated chemokine
TB	Tuberculosis
TCR	T cell receptor
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial cell growth factor
VLA	Very late antigen
VZV	Varicella voster virus
WBC	White blood cell

Publications, presentations and prize list

PUBLICATIONS

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PRESENTATIONS

Local/ Regional Oral Presentations:

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International Oral Presentations:

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1. Introduction

1.1 The skin and the immune system.

The skin is the largest organ of the body and has a critical role in acting as a barrier to the environment in addition to maintaining homeostasis by preventing water loss and regulating body temperature. As well as providing a mechanical barrier to pathogens, the skin has a highly specialized immune system in order to respond to the wide variety of antigens that it constantly encounters. Cells capable of both innate and adaptive immune responses are resident within the skin and can be recruited into the skin at sites of inflammation (Table 1.1), allowing for the provision of immuno-surveillance and protective immunity. In particular, these different cell types play an important role in the initiation and amplification of immune responses, with close interlinking between innate and adaptive pathways.

Several cell types present in the skin are organ-specific, making cutaneous immune responses unique. The epidermis is densely populated by antigen presenting cells known as Langerhans cells that are thought to play a major role in primary immune responses in the skin. In addition, the skin has organ-specific T lymphocytes, identified by certain cell surface molecules, which are thought to re-circulate between lymph nodes and skin. Constantly exposed to light, the skin also has specialized cells (melanocytes and keratinocytes) that are highly resistant to the DNA damaging effects of ultraviolet irradiation

TABLE 1.1 Cells of the Adaptive and Innate Immune system

	RESIDENT	**RECRUITED**
ADAPTIVE	T lymphocytes	T lymphocytes
		B lymphocytes
INNATE	Dendritic cells • Langerhans cells • Dermal dendritic cells	Dendritic cells • Plasmacytoid dendritic cells
	Keratinocytes	Monocytes
	Endothelial cells • Vascular • Lymphatic	Granulocytes • Neutrophils • Basophils • Eosinophils
	Mast cells	Epithelioid cells
	Tissue macrophages	

1.2 T cell mediated immune responses

1.2.1 Primary immune responses

Primary T cell responses are mediated by naïve T cells that, after leaving the thymus, circulate between the blood and secondary lymphoid organs including the lymph nodes, spleen and Peyer's patches[1;2]. Naïve T cells express high levels of L-selectin and CCL7, the ligands for the vascular addressins (CD34 and GlyCAM-1) and secondary lymphoid tissue chemokine (SLC) respectively, which enables them to enter the lymph nodes via the high endothelial venules[3].

Initiation of a primary immune response is thought to occur within the secondary lymphoid tissue following naïve T cell recognition of processed peptide antigen complexed to major histocompatability (MHC) molecules on the surface of antigen-presenting cells (APCs)[4;5]. Recognition of antigen results in the triggering of the T cell receptor (TCR)/ CD3 complex as well as CD4 or CD8 co-receptors with the formation of an immunological synapse between the T cell and APC. Cell triggering is also dependent on the ligation of additional co-stimulatory, accessory and adhesion molecules such as CD28, LFA-1, CD40L, OX40 that serve to augment T cell activation and expansion by stabilising the immunological synapse and inducing intracellular signalling molecules[6-9]. Naïve T cells subsequently undergo rapid proliferation and differentiate into effector cells.

Following activation, some CD4+ T cells will migrate towards the B-cell follicle in which they are activated and provide help to B cells[10]. Both CD4 and CD8 effector T cells will also localize to the extralymphoid tissues via the efferent lymph vessels and thoracic duct where they will be involved in immune responses in the periphery. Effector T cell subsets show tropism for different tissues[11] such as the skin and this is thought to be determined by the induction of tissue-homing receptor expression following priming in addition to the expression of ligands for the receptor in the skin vascular bed.

The induction of tissue homing receptors specific for a certain tissue type is thought to depend on lymph node environment and the dendritic cells derived from the tissue in which antigen is encountered[12]. The cutaneous lymphocyte

antigen (CLA) is thought to identify a subgroup of effector T cells with preferential capacity for homing to cutaneous tissues whereas $\alpha 4\beta 7$ integrin expressing cells home to the intestine[13]. CLA is a ligand for both endothelial cell selectin (E-selectin) and platelet selectin (P-selectin) which are expressed constitutively on dermal microvessels and whose expression is up regulated following cutaneous inflammation[14-17]. Studies have shown that CLA expression is preferentially expressed on T cells during naïve T cell activation in skin-draining lymph nodes, demonstrated in mice with the use of TCR transgenic T cell adoptive transfer models[15;18]. In addition, skin derived antigen presenting cells (APCs) are also thought to play a crucial role in that Langerhans cells have been shown to be more effective in generating skin-tropic effector cells than peripheral lymph node dendritic cells[12].

During a primary immune response the increase in the number of cells within inflamed non-lymphoid tissue is thought to be dependent on the recruitment and retention of cells derived from clonal expansion within the lymph node, rather than as a result of *in situ* proliferation[19;20]. Effector T cells attracted to the site of inflammation are able to directly or indirectly eliminate the foreign antigen by the production of various cytokines and other cytotoxic agents. CD8+ T lymphocytes have direct cytotoxic activity through the secretion of perforins, granzymes and cytokines such as interferon gamma (IFN-γ) and tumour necrosis factor (TNF). CD4+ effector T lymphocytes provide help to enhance CD8+ T lymphocyte differentiation and activate macrophages and B cells. Activation of effector CD4+ cells at the site of inflammation results in cellular differentiation with resulting polarised patterns of cytokine production. IL-12 and IFN-α promote the development of Th1 polarised cells that secrete IFN-γ and TNF whereas IL-4 promotes the generation of Th2 polarised cells that secrete IL-4, IL-5 and IL-10[21;22]. Some CD4+ T cells secrete both types of effector cytokines and are known as Th0 cells. In general, Th1 responses are thought to be protective against intracellular infections whereas Th2 responses are effective against extracellular infections.

Following clearance of antigen, there is subsequently a contraction phase where there is a dramatic reduction in the numbers of T cells. The reduction in the numbers of T cells present at the site is thought to involve multiple pathways, resulting predominantly in apoptosis[23-26], either as a result of activation induced cell death[24;25] or cytokine deprivation[27]. A few antigen-specific cells survive however and become long-lived memory T cells. The precise pathway by which effector T cells become memory T cells remains unclear although a linear model of differentiation has been proposed whereby a subset of effector cells escape apoptosis and revert to a quiescent group of memory cells[28;29].

1.2.2 Memory T cell responses

Memory or recall responses occur more rapidly and are more pronounced than primary responses[28;30]. This may be due to an increase in the frequency of antigen specific memory T cells within the T cell pool compared with naïve cells. However, it is also thought that memory T cells have a reduced requirement for activation in that they respond to lower concentrations of antigen, use less co-stimulatory signals, need a shorter duration of antigenic stimulation[31] and enter in to cell cycle more rapidly following TCR stimulation[32]. Memory T cells also express different patterns of adhesion molecules and chemotactic receptors, enabling them to interact with APCs more effectively and also to migrate in to inflamed non-lymphoid tissues. Memory T cells differ from naïve T cells in their tissue distribution and circulation pattern. They can be found in both lymphoid and non-lymphoid sites[33;34] and are readily activated by antigen in all tissues[35-37]. Tissues such as the liver, lung and gut are major reservoirs of antigen experienced T cells.

1.2.3. Memory T cell phenotype

CD45, the leukocyte common antigen, is used to differentiate naïve from effector and memory T cells. The relative expression of the CD45 isoform can be used to distinguish the state of T cell differentiation[38]. CD45 is a receptor like protein tyrosine phosphatase (PTP) and is expressed on all nucleated haematopoietic cells including leucocytes[39]. The precise role and ligand for

the receptor have yet to be fully determined[40] however CD45 appears to play an important role in T cell signal transduction[41;42]. Naïve T cells express the CD45RA isoform, however following activation the expression of CD45RO is rapidly upregulated and CD45RA expression is lost over a few days[43]. All CD45RA+ naïve T cells also express high levels of CD45RB. CD45RO+ T cells can be divided in to CD45RB high and low subsets depending on whether they have recently been primed or have undergone numerous rounds of stimulation and are highly differentiated respectively[44]. The loss of the co-stimulatory molecules CD27 and CD28 on memory T cells can also be used to identify cells with a more differentiated phenotype[45].

Recent studies have shown that human memory CD8+ T cells can re-express CD45RA and lose CD45RO expression in a process known as reversion[46-48]. CD8+ revertant T cells, unlike naïve T cells, express low levels of L-selectin (CD62L) and CCR7 but high levels of LFA-1. Limited evidence also suggests that CD4+ T cells may also revert to CD45RA+ phenotype[49]. The precise role of revertant memory cells has yet to be identified but they may form a more stable, non-cycling memory population.

CD45RA- memory T cells can be further divided on the basis of expression of CD62L and CCR7[50]. Central memory cells express both CD62L and CCR7 and are located primarily in secondary lymphoid organs. Although these cells lack immediate effector function, they proliferate well, produce IL-2, efficiently stimulate dendritic cells and differentiate into CCR7- effector cells upon secondary stimulation. In contrast, effector memory T cells do not express CD62L or CCR7[51],express tissue homing receptors and can reside in both secondary lymphoid tissues as well as non-lymphoid tissues[52]. These cells have been found to rapidly secrete effector cytokines IFN-γ, IL-4 and IL-5 after restimulation[53] but may not proliferate as efficiently as the central memory T cells. It has therefore been proposed that the effector memory subset is specialised for quickly entering inflamed tissues in order to provide immediate effector function whereas the central memory subset is responsible for a more sustained memory response.

1.2.4 Delayed type hypersensitivity responses and the skin

Delayed type hypersensitivity (DTH) responses in the skin can be induced by either the topical application of hapten or the intradermal injection of antigen in to the skin and are representative of a cutaneous T cell mediated memory response[384]. The delayed type hypersensitivity response to the intradermal injection of bacterial, viral and fungal antigens can therefore be used to assess individuals' cell-mediated immune responses to particular antigens. A positive skin test denotes prior antigenic exposure, T cell competency and an intact inflammatory response.

Responsiveness to DTH skin testing is assessed by the diameter of induration induced in the forearm skin in humans at 48 hours or by the amount of ear or footpad swelling at 24 hours in mice. In humans, following the injection of intradermal antigen, a clinical response is seen within a few hours with white or rose-coloured induration of the skin. The visible response in the skin peaks by 48-72 hours with a localized red, indurated area of skin. Rarely, vesicles and petechiae may also be seen. Ulceration at the site of injection has also been reported. The clinical response then reduces and resolves by 10-14 days[384].

DTH responses are used clinically to detect either infection with or previous exposure to various pathogens including *Mycobacterium tuberculosis*. The classical model of DTH is the Mantoux test where a sterile aqueous solution of tuberculin purified protein derivative, a complex mixture of peptides and carbohydrates derived from *M. tuberculosis*, is injected intradermally in to the skin[384]. It is used widely as an experimental model in that it produces a reproducible clinical response in the skin in young, healthy individuals previously exposed to *M. tuberculosis* and those immunized with the Bacillus of Calmette and Guerin (BCG) vaccine[54], prepared from live attenuated strains of *M. bovis*[55]. The likelihood of previous immunization versus acute infection is determined by the diameter of the clinical response with a diameter of over 15mm regarded as suspicious of current infection[56], warranting further investigation (NICE guidelines, March 2006).

DTH responses against common pathogens such as Candida or vaccine antigens (Tetanus) can also be used clinically as a measure of overall cell mediated immunity. The Candin skin test, using a sterile solution produced from the culture filtrate and cells of two strains of *Candida albicans*, has been licenced for the evaluation of cellular immune responses in the skin in patients with HIV and is reported to have prognostic value in cancer[57-59].

Histological analysis indicates that the DTH response in the skin is biphasic with an early non-specific infiltration of cells that occurs in all individuals and a second specific peak that is only seen in those who have previously been sensitized to the antigen[60;61]. Initially, at around 4-6 hours there is an infiltration of neutrophils in to the skin[61] followed by macrophages that peak in numbers at around 24-48 hours[62]. Around 12 hours after challenge T lymphocytes and dendritic cells have been shown to appear around dermal blood vessels[61]. After 48 hours the majority of infiltrating cells are T lymphocytes with CD4+ lymphocytes exceeding CD8+ lymphocytes at all time points[62;63]. Only a very small number of B lymphocytes have been observed at the site of DTH responses[61-63]. The majority of cells are seen to accumulate perivascularly, however T cells and macrophages are also found within the interstitium in the upper dermis and also around adnexal structures such as hair follicles and sweat glands[61-63]. Lymphocytes may also be seen infiltrating the epidermis[61-63].

Previous investigations have focused on the histological findings during the course of the clinical response up to around 96 hours however we have observed that while the peak clinical response occurs at 3 days, the peak of lymphocytic infiltration is not reached until day 7[64]. Thus, many studies have overlooked the fact that significant T cell reactivity persists beyond time points where a visible clinical response is seen in the skin.

1.2.5 Initiation of secondary immune responses in the skin

The pathways involved in the elicitation of a cutaneous memory immune response remain poorly defined. Studies have shown that large numbers of both CD4+ and CD8+ T lymphocytes are resident within normal skin[65;66]. In

addition, the vast majority of (skin homing) CLA+ memory T cells within the body are thought to be located within the skin[65]. Schaerli et al further characterised the resident skin population and found that the most T cells resident in the skin express CCR8 and they proposed that these cells act as immune surveillance cells[66].

The conventional model of DTH responses in the skin suggests that lymphocytic recruitment to the skin occurs following secondary lymph node T cell proliferation after migration of antigen presenting cells from the skin to the draining lymph node. Although Langerhans cells are conventionally regarded as the main antigen presenting cells of the skin[67], it is unknown whether they are able to take up and process antigen that has been injected intradermally. Their contribution to the DTH response is therefore unclear. Another population of dendritic cells in the dermis, known as dermal dendritic cells, may play an important role in DTH responses[68]. Other cells within the skin also have antigen-presenting capability such as macrophages[69] and keratinocytes[70-73] and may play a more prominent role in antigen presentation following secondary immune challenge. Human dermal microvascular endothelial cells (DMEC) may present antigen to circulating memory cells[74;75]. DMEC express high levels of MHC class 1 and class 2 molecules and can induce human CD4+ and CD8+ memory but not naïve T cells to proliferate and secrete cytokines *in vitro*.

It is possible that interactions between resident T cells and antigen presenting cells are responsible for the initiation of DTH responses in the skin without the need for the initial migration of dendritic cells to the lymph nodes. Although T cell activation has been observed in draining lymph nodes following secondary challenge, this occurs at a reduced rate compared with the primary response[76]. Based on calculations of circulating volumes and cardiac output it has been estimated that the time taken for antigen to reach the inguinal lymph nodes from the toe would take around 12 hours[77], however DTH responses are seen as early as 4 hours. In addition, recall immune responses in humans occur in spite of compromised afferent lymphatics[78]. All of these findings

suggest that localised initiation of the immune response may occur within the skin.

The close apposition of antigen presenting cells and T cells within the skin during recall responses[79;80] may indicate that the activation of resident antigen- specific T cells by antigen presenting cells within the skin is possible. Activation of antigen specific cells within the skin could potentially result in the initiation of the immune response via the secretion of acute phase pro-inflammatory mediators such as TNF-α and IFN-γ resulting in the activation and recruitment of monocytes, neutrophils and memory T cells. Resident T cells may also be able to traffic to the lymph node where they are able to accelerate appropriate T and B cell responses.

One of the problems with this model however is the large diversity of antigen-specific cells required in order to accommodate the vast array of antigens that are encountered by the skin. Antigen specific cells may only be resident at the sites of previous antigenic challenge. This theory is supported by the observation that repeated allergen exposure to a site of previous allergic contact dermatitis in guinea pigs results in a more rapid clinical response, not seen at other non-exposed sites[81]. In addition, in humans, IFN-gamma producing CD8+ T cells have been found in the epidermis of fixed drug eruptions before and after secondary challenge[82].

1.2.6 T cell proliferation and clonal expansion during a memory recall response.

As with primary immune responses, expansion and then subsequent contraction of T cell numbers occurs during the course of the DTH response at the site of inflammation. Although studies in mice have demonstrated the persistence of memory T cells within non-lymphoid tissues that appear to convey protective immunity[33;83], other studies have shown that memory T cells resident within non-lymphoid tissues do not proliferate upon reactivation[84;85]. Blocking chemokine or integrin mediated recruitment of T lymphocytes during DTH responses in mice diminishes the response[86;87] suggesting that

recruitment of T cells within the circulation or resident within secondary lymphoid tissues is critical in mediating a recall response. In mice, therefore, the increase in the number of lymphocytes at the site of secondary immune responses is unlikely to be due to proliferation of antigen-specific cells within non-lymphoid tissues.

Published work from our laboratory, however, suggests that the expansion of PPD specific CD4+ T cells during the course of the Mantoux test response in humans is due to the *in situ* proliferation of T cell clones present in the skin at early time points[88]. This conclusion is supported by the findings that there was a high level of the proliferation marker Ki67 present in the skin around Day 7 and that there was an increase in the number of antigen specific cells up to day 14. However, there was no associated change in the number of circulating Ki67 or PPD-specific T cells in the peripheral blood that would be expected if the T cells were recruited to the skin following proliferation in the lymph node. Heteroduplex analysis also indicated that similar clones were present at early and late stages of the response[88].

DTH responses may therefore be mediated by several mechanisms in different species, some of which may become redundant under certain conditions, including activation of resident cells and the recruitment of memory T cells from the peripheral blood some of which will have been pre-activated in the draining lymphoid tissue.

1.3 Innate Immunity

Innate immunity is an evolutionarily ancient component of host defense mechanisms[89] and provides the first line of defense against pathogens using pattern recognition receptors (PRRs)[90], a group of which are known as Toll-like receptors (TLRs)[91].

1.3.1 Innate immunity and the skin

The skin is an important part of the innate immune system, forming a barrier to prevent the entry and colonization of pathogens. The epithelial cell membranes express PRRs that are capable of responding to pathogen

associated molecular patterns (PAMPs) by secreting a variety of antibacterial peptides including defensins, cathelicidins and dermicidins[92;93]. The absence of defensins in the skin has been proposed as a mechanism for recurrent infection seen in atopic dermatitis[94]. In addition, constant shedding of the epithelial cells along with the secretion of sebum, lysozyme and mucins helps to reduce bacterial burden on the skin surface.

If the skin's integrity is breached then macrophages have an important role to play in detecting danger signals via their PRRs either from PAMPs or endogenous signals released in damaged tissue[95-97]. Macrophages act to phagocytose invading organisms and are able to present antigen in the context of MHC. They also secrete cytokines, resulting in the recruitment and activation of neutrophils and natural killer cells and the induction of maturation, differentiation and migration of dendritic cells. This then allows for the development of an adaptive immune response with subsequent clearance of the pathogen. Professional antigen presenting cells in the skin, Langerhans and dermal dendritic cells, are also able to become activated at the site of injury or pathogen invasion through PRRs[98]. Both types of dendritic cell are thought to serve to amplify the immune response by the production of pro-inflammatory cytokines but also process antigen and emigrate to the draining lymph node where antigen is presented to antigen-specific T cells.

1.4 Chemokines and cytokines

Cytokines and chemokines play a crucial role in the body's defence against pathogens. In the early stages of infection a family of chemoattractant cytokines, known as chemokines, are secreted by a variety of cells within the affected tissue. They act as chemoattractants for leukocytes[99] such as monocytes and neutrophils, in addition to functioning in lymphocyte development and migration. Chemokines contribute to immune response initiation, influencing cells involved in both innate and adaptive immune responses, and may also have a role in immune pathology. In addition to their involvement in inflammation, chemokines also have an important role to play in homeostasis and immune surveillance of healthy tissues. Chemokines fall

in to 2 main structural groups: those characterised by a CC structure with two cysteine residues near the amino terminus and CXC chemokines in which the two cysteine residues near the amino terminus are separated by another amino acid.

1.4.1 The role of cytokines and chemokines in immune responses in the skin.

Cytokines and chemokines are thought to be involved in all phases of inflammation in the skin. Multiple cell types in the skin, including keratinocytes, T and B lymphocytes, monocytes, endothelial cells, fibroblasts, mast cells, dendritic cells, granulocytes and platelets are known to produce cytokines. The initiation of the response to infection in the skin, known as the acute phase response, is thought to be dependent on the production of tumour necrosis factor (TNF), IL-6 and IFN-γ[383]. TNF is secreted by a variety of cells including monocytes and macrophages, T cells, fibroblasts and endothelial cells. Following stimulation by exogenous factors such as bacterial lipopolysaccharide, viruses and other organisms large quantities of TNF are released within minutes and synthesis is rapidly increased under the influence of IFN-γ[100].

TNF acts to increase vascular permeability and blood flow resulting in the observed erythema and induration at the site as well as increasing expression of various adhesion molecules E-selectin, Intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM) on the endothelium[101] with resulting recruitment of lymphocytes to the site of the immune response and also activation and chemotaxis of neutrophils and monocytes[102]. IFN-γ produced predominantly by activated T lymphocytes increases MHC- II expression on monocytes and macrophages[103] in addition to stimulating further release of TNF-α by monocyte activation[104]. IL-6 is produced by activated monocytes and is important for T cell activation and differentiation[105;106].

Following acute initiation of the immune response in the skin further cellular activation and recruitment is mediated by the production of a range of cytokines and chemokines from several resident skin cell types, as shown in Table 1.2. MCP-1, MIP 1-α and RANTES are classified as CC chemokines and are thought to have an essential role in humans in the activation and chemoattraction of monocytes to sites of inflammation. The CXC chemokines, such as IL-8 and IP-10, are thought to attract and activate neutrophils[107]. A mixture of both CC and CXC chemokines have been shown to have an affect on the induction of directional migration of human T cells and T cell subsets including memory effector T cells.

TABLE 1.2 Chemokines and the skin.

Chemokine	Produced by	Receptors	Cells attracted	Cells activated
CCL17 (TARC)	Endothelial cells	CCR4	T cell Dendritic cell NK cell	T cell
CCL27 (CTACK)	Keratinocytes	CCR10	T cell	
IP-10	Keratinocytes Fibroblasts	CXCR3	T cell	
MCP-1	Macrophages Monocytes Keratinocytes Endothelial cells Fibroblasts	CCR2B	Monocytes T cells Dendritic cells NK cells Basophils	Macrophages Fibroblasts Endothelial cells
MIP 1α	Monocytes T cells Fibroblasts Dendritic cells	CCR1, 3, 5	Monocytes T cells Dendritic cells NK cells Basophils Neutrophils	Mast cells Basophils
RANTES	T cells Endothelial cells Platelets Macrophages Keratinocytes Fibroblasts Dendritic cells	CCR1, 3, 5	Monocytes T cells Dendritic cells NK cells Basophils Eosinophils	T cells Basophils
IL-8	Monocytes Macrophages Keratinocytes Fibroblasts Endothelial cells Mast cells	CXCR1, 2	Neutrophils	Neutrophils

1.4.2 Skin related chemokine receptors and their role in lymphocyte homing into the skin.

A number of chemokines and their receptors are associated with the homing of T cells to the skin. The initial process of lymphocyte recruitment is dependent on the interaction between adhesion molecules expressed on the surface of both lymphocytes and the endothelium of the dermal microvasculature. This process can be broken down in to 3 main steps: rolling, adhesion and transmigration. Due to the sheer forces encountered within the blood stream, lymphocytes do not bind to resting endothelium. However, in inflamed skin the expression of selectins, a group of carbohydrate mediated adhesion proteins, on endothelium allow for transient reversible binding of endothelial cells with inflammatory T cells. This results in a process known as rolling where the inflammatory cells are slowed and are seen to move in a linear direction, rolling along the endothelium. In the skin, E-selectin is thought to be the most important selectin to be expressed on endothelial cells[108], the upregulation of the expression of which is thought to be mediated by TNF and IFN-γ[17;109]. Memory T cells localizing to the skin express the cutaneous lymphocyte antigen (CLA) which functions as a ligand for E-selectin[110]. CLA expression on the cell surface arises from the specialized glycosylation of P-selectin glycoprotein ligand 1[111;112] and is found only on T cells that have undergone primary activation in the draining lymph nodes of the skin[18]. While only 10-25% of T cells in the peripheral blood express CLA, 80-90% of cells at sites of cutaneous inflammation express this marker[113] indicating that CLA expression plays a major role in the selective recruitment of skin-specific T cells in to the skin.

After selectin mediated rolling on the endothelium, lymphocytes become activated and firmly attach to the endothelium through the adhesion of beta 2 integrins to LFA-1 and VLA-4 and the immunoglobulin superfamily members ICAM- 1, 2 and VCAM-1 that are expressed on the endothelial surface[114]. The expression of endothelial ICAM and VCAM is upregulated in inflamed skin, induced by IL-1[115], IFN-γ[116] and TNF-α[115].

In inflamed skin, certain chemokines are bound to the endothelium and interact with receptors on the lymphocyte cell surface, resulting in a morphological change in integrin receptor affinity and avidity for their ligands, promoting endothelial adherence. Further lymphocyte adhesion to endothelium and subsequent transendothelial migration of CLA+ T cells in the skin is thought to be critically dependent on the interactions between the chemokines CCL17 (TARC) and CCL27 (CTACK) with their receptors CCR4 and CCR10 respectively[87]. CCR4 and CCR10 seem to have redundant and overlapping roles in mediating the recruitment of T cells in to inflamed skin. While CCR4 is expressed on >75% of CLA+CD4+ T lymphocytes, CCR10 is only expressed on around 28% of CLA+ memory T lymphocytes[117]. In addition virtually all CCR10+CLA+ T lymphocytes also express CCR4[117]. CCL17 (the ligand for CCR4) expression is found predominantly on dermal vascular endothelium, whereas CCL27 (the ligand for CCR10) is produced mainly by keratinocytes in the epidermis[87]. The expression of these ligands within the skin matches the location of T cells bearing the appropriate receptors in that CCR4+ T cells are found in the dermis[118] and CCR10+ T cells are found in the dermis and epidermis in inflamed skin[119]. CCR4 has an additional role in the initiation of immune responses as it is thought to be important in the mediation of dendritic cell and memory T cell interactions via the ligand macrophage derived chemokine (MDC) in both the dermis and secondary lymphoid tissues[120;121].

Although it is not tissue specific, CXCR3 is thought to be an important skin homing receptor. Predominantly expressed on memory T cells, CXCR3 expression is thought to define a subset of memory T cells homing to sites of Th1 inflammation including the skin, intestine and CNS[122]. The importance of CXCR3 in skin inflammation is highlighted by the fact that a significant subset of CXCR3 positive CD4+ T cells also express the skin specific receptor CCR4[123]. CXCR3 is the receptor for the inflammatory chemokines IFN-γ inducible protein (IP-10), monokine induced by IFN-γ (Mig) and IFN T cell α chemoattractant (I-TAC)[124]. Several skin cell types including keratinocytes, monocytes and fibroblasts have been shown to produce IP-10 and I-TAC,

stimulated by IFN-γ[125]. CXCR3 may have a role in pathological inflammation both in the skin and other organs such as the liver, with upregulation of the receptor on T lymphocytes at sites of chronic inflammation[126]. In inflammatory skin disease, it has been shown that greater than 75% of cells express CXCR3. In addition, the pattern of T cell infiltration in different skin diseases is dependent on the location of expression of ligands for CXCR3 within the skin [127].

Recent work has shown that the majority of resident skin T lymphocytes are CCR8 positive and respond to its selective ligand CCL1 (I-309)[66]. It is thought that CCR8/ CCL1 interactions may be crucial for immunosurveillance and homeostatic T cell trafficking in normal skin as CCL1 is expressed on dermal endothelial cells in addition to epidermal Langerhans cells in normal, uninflammed skin[66]. The role of the CCR8/ CCL1 axis in the recruitment of cells during acute inflammation is currently unclear although it is of interest that CCR8+ T lymphocytes have a pre-activated (CD69+) phenotype and have been shown to secrete the pro-inflammatory cytokines TNF-α and IFN-γ[66], both of which are crucial for the acute phase response in memory recall responses in the skin. In addition, the CCL1-CCR8 axis has been implicated in atopic skin inflammation[128].

1.5 The effects of ageing on immunity

1.5.1 The impact of ageing on health.

Over the last century human life expectancy has increased dramatically in developed countries. The number of people aged 60 years or over in the UK is projected to increase from 12 million (20%) of the population in 2001 to 18.6 million (30%) in 2031[129] (National office for statistics). This is likely to place significant demands on the provision of healthcare as many diseases in the elderly are more frequent and severe. In particular, infectious diseases are seen more commonly in the elderly and are often associated with poor outcome with higher rates of morbidity and mortality[130]. The immune system is thought to decline with ageing although the relevance of this to the development of disease has not been fully characterized[131]. The concept of

the immune risk phenotype (Table1.3) has emerged as a result of long- term studies[132-135] that have attempted to identify serological markers of immune function thought to be predictive of poor health with increased mortality and morbidity.

TABLE 1.3

Immune –risk phenotype:

- CD4/CD8 ratio less than 1
- Poor T cell proliferative response
- Low levels of B cells
- Increase in terminally differentiated memory T cells
- CMV positivity
- Increased level of IL6 and TNF-α

Pyogenic bacteria are the most common cause for infection in the old causing urinary tract infection, pneumonia, diverticulitis, endocarditis, bacteraemia and skin and soft tissue infection[136]. Previous studies have shown three- fold increases in the prevalence of community acquired pneumonia[137] and a 20 times increase in urinary tract infection in the old[138]. The clinical presentation of very old patients with bacterial infection also differs from presentation in the young with fewer symptoms such as reduced or absent fever in around 20-30% of severe infections[139]. Certain viral infections are also more common, in particular influenza[140], herpes zoster reactivation (shingles)[141] and viral gastroenteritis[142]. Unfortunately vaccinations, such as those used to protect against influenza virus infection, have been shown to have a reduced efficacy in the old[143]. It has also been proposed that infections themselves may lead to the development of age related diseases such as atherosclerosis[144;145] and dementia[146;147]. Recent work has shown that chronic infections with latent infections such as CMV have potentially deleterious effects on the immune system, resulting in so-called immunosenescence[132].

An increase in certain types of cancer affecting various organs is seen with advancing age, in particular carcinomas[148;149]. In the skin, the number of cancers is well recognized to increase with age. The number of recorded non-melanoma skin cancers in the UK in 2001 was around 62,700 of which 80% of the cases occurred in individuals aged 60 and over[150]. Autoimmunity has also been shown to increase with ageing[151] with increased production of autoantibodies and the development of late onset diseases such as thyroid disease, pulmonary fibrosis and Sjogrens syndrome[152].

1.5.2 Effects of ageing on T lymphocytes

Although there is evidence to suggest that changes in several immune cell types occur with ageing, certain specific observations suggest that T cell memory responses are impaired with ageing. In particular, the reactivation of latent organisms such as VZV[153], EBV and mycobacteria[154] occurs more frequently with age. Reactivation of VZV and reduction in immunity following smallpox vaccination has been shown to be correlated with a decline in antigen- specific memory T cell number rather than reduced antibody levels[155;156] suggesting that T but not B cells are impaired. In the skin, there are several studies that have shown a reduction in the DTH response, a classical memory T cell response, following the injection of numerous antigens including tuberculin purified protein derivative, tetanus toxoid, candida, trichophyton and mumps in the old[157-161]. DTH responses to the topical application of various haptens have also been shown to be reduced, with higher doses of hapten required for the development of contact hypersensitisation[162-165].

In early life, the thymus is thought to play an important role in the generation of naïve T cells, however the thymus involutes with ageing and is almost completely replaced by fat by the age of 60[166]. In spite of involution of the thymus, the absolute number of T lymphocytes remains relatively stable with age[167] indicating that the population of T cells is maintained by turnover of pre-existing populations of cells. However, the functional quality and proportions of antigen-specific cells can be dramatically altered with age resulting in a decline in overall immune function and a potential loss of

immune memory to certain types of infection. Old individuals have significantly higher numbers of T cells specific for certain types of chronic, persistent viruses with massive clonal expansion of cells specific for infections such as CMV[168-170]. However, these cells have a reduced functional capacity[171-173] and may result in the crowding out of T cells specific for other infections and a resulting loss in T cell receptor diversity[174].

The functional capacity of T cells is reduced with ageing. Numerous studies in the old have shown reduced proliferative capacity of T lymphocytes to various mitogens including PHA, Con A and SEB[175-179]. A reduction in proliferative capacity has also shown to be linked to mortality and morbidity in the populations studied[180]. T cell proliferation requires the presence of IL-2 and IL-2 receptors both of which have consistently been shown to decline with ageing[181;182]. In addition, increased production of IL-10 in the old[175;183] may act to suppress proliferative responses[184]. IL-10 neutralizing antibodies have been shown to restore proliferative responses of elderly PBMCs to responses equivalent to those seen in their younger counterparts[175]. Increased production of IL-6 has also been observed in the old and is associated with increased mortality[135].

It has been proposed that there is a shift in Th1 to Th2 type T cell responses in the old[185]. Whereas Th1 cells produce cytokines favouring a cell-mediated response, in particular IL-2 and IFN-γ, Th2 cells produce IL-10, IL-4, IL-5 upon activation favouring humoral responses. The shift in cytokine production may help to explain the apparent reduction in cell-mediated responses in the old with increased autoimmunity due to the augmentation of B cell mediated antibody production. However, although consistent results have been observed for reduced IL-2[181;182] and increased IL-10 production[175;183] in the old, variable reports of IFN-γ and IL 4 production have been cited according to the model and the type of stimulation used. Studies in aged mice have shown reduced production of IFN-γ with Con A stimulation but increased or normal levels following stimulation with phorbol esters[131]. In human studies using

both healthy and frail elderly -subjects both increased and decreased production of IFN-γ[185] and IL-4[185] have been reported.

T cells also have been shown to have alterations at a biochemical and molecular level with ageing. There is a decrease in transmembrane signalling following activation of the T cell receptor, thought to be due to a decrease in protein tyrosine phosphorylation and generation of second messengers, calcium ion mobilization and translocation of protein kinase C[182;186-189]. In addition there is an observed reduction in the induction of transcription factors NFAT and NF kappa B following T cell activation which may explain the observed decreased expression of several cytokines. Whisler et al showed that the decreased expression of IL-2 by T cells correlated with impairment in the activation of AP-1 and NF-AT transcription factors[182]. Increased oxidative stress following the generation of reactive oxygen species which results in increased DNA damage and alteration may also affect T cell function with ageing[190].

The proportions of highly differentiated effector memory T cells are significantly increased in the old in comparison to younger subjects[191;192]. Recent research has also focused on the accumulation of so- called revertant cells in the old[191]. These CD45RA+ cells are thought to accumulate in response to chronic infection with CMV[170] and EBV[193], and their numbers are significantly increased in both the CD4+ and CD8+ pools of old individuals[191].

All normal somatic cells in humans, including T lymphocytes, are able to divide a finite number of times and this is known as replicative senescence[194-196]. Telomere length can be used to identify cells at different stages of differentiation due to cellular division[197;198]. Telomeres are repeating hexameric sequences of nucleotides at the end of chromosomes that shorten by 50-100 base pairs per cell division due to the inability of DNA polymerase to fully replicate the ends of chromosomes during cell division. Telomere length can be restored by the enzyme telomerase[199-202], however shortening

of telomere length eventually leads to chromosomal instability, end to end fusions and cell growth arrest or apoptosis.

Highly differentiated memory T cells have short telomere lengths, lose telomerase activity and have a reduced capacity to divide[202]. Their persistence *in vivo* is therefore likely to be limited. DNA derived from blood and skin has been shown to have overall shorter telomere lengths in the old[203]. Shortening of telomere length may in part be due to downregulation of costimulatory CD28 receptor in memory T cells as upregulation of telomerase is dependent not only on TCR stimulation but also transduction of costimulatory signals through CD28[202]. Thus chronic activation of T cells due to ageing, persistent viral infection or repeated exposure to antigen may result not only in loss of CD28 expression but also loss of telomerase activity and shortening of telomere length.

Telomere shortening has been demonstrated with ageing in both B and T lymphocytes with a loss of around 34-54 bp per year[204]. There is good evidence to suggest that telomere erosion has a negative impact on immunity in addition to mortality and age related disease[205-207]. This is highlighted by the syndrome known as Dyskeratosis Congenita (DKC) where patients have deficient telomerase activity and an associated reduction in peripheral blood mononuclear cell (PBMC) telomere length compared with individuals of a similar age[208;209]. 83% of individuals with this disease have absent or reduced cutaneous DTH reactions[210] suggesting that T cell memory is compromised[211]. In addition, Werners Syndrome is characterised by premature ageing of the skin related to telomere dysfunction in fibroblasts[212;213].

In spite of the potential complications of telomere shortening seen in association with ageing T lymphocytes, down-regulation of telomerase may also have an important beneficial role during the course of acute inflammatory responses. We have previously shown marked telomere shortening of PPD specific cells in the skin, but not blood, during the course of a DTH response to PPD in young individuals[88]. Telomerase activity in these cells was found to

be inhibited by IFN-α produced within the microenvironment of the skin. Removal of cells from the skin allowed for reversible reactivation of telomerase activity, suggesting that maintenance of the memory T cell population may be enabled by upregulation of telomerase once cells had emigrated from the skin. This mechanism may be important for limiting uncontrolled T cell proliferation during secondary immune responses. In the old, inhibition of telomerase may have a beneficial effect by helping to balance the pro-inflammatory *milieu* seen with ageing, referred to by some authors as inflamm-ageing[152].

1.5.3 Effects of ageing on innate immunity

Although research in the past has focused on adaptive immune dysfunction with ageing, there is now increasing evidence to suggest that cells involved in innate immunity may also be affected by ageing[214] and that impaired innate immunity may predict frailty in old age[215]. Stimulation of whole blood with LPS in the old results in low production of pro-inflammatory cytokines, indicating that certain cell types involved in innate immunity may become defective with ageing[215].

Several changes in monocytes and macrophages have been identified with ageing[216]. Although the number of blood monocytes in the old and young is similar, there is a decline in the numbers of macrophages and their precursors in the bone marrow of the elderly[217;218]. Ageing human and rodent macrophage populations have reduced MHC-II expression[219;220] and it has been suggested that this results in poor T cell responses. Defects at the level of TLR expression and function on macrophages have also been demonstrated in murine models. Renshaw et al demonstrated a reduction in TLR4 expression and other TLRs in mice[221] although other more recent studies have been unable to replicate these findings[222]. Defects in TLR signaling pathways have also been demonstrated in mice including defects in the protein kinases mitogen activated protein kinase (MAPK) p38 and c-Jun N-terminal kinase (JNK)[222]. Animal models have shown a reduction in macrophage adherence[223], opsonisation and tumour cell killing with age[224;225].

In old humans with chronic bronchitis macrophage phagocytosis is also reduced[226]. The decline in phagocytic capacity of macrophages is thought to occur in parallel with a reduction in the production of macrophage-derived chemokines MIP-1α, MIP-1β and MIP-2 in a model of dermal injury[227].

The phagocytic capacity, synthesis of reactive oxygen intermediates and intracellular killing efficiency of neutrophils is impaired with ageing[228]. In addition, NK and NKT cell production of cytokines such as IFN-γ and chemokines are reduced with age[229]. Ogata et al have demonstrated an association between low NK cell activity and infection with short life expectancy due to infection[230].

Shifts in the production of cytokines in the elderly may alter the maturation and balance of DC subsets. In particular, the documented increase in IL-10 production in old may affect the maturation of DC subsets. It has also been proposed that a reduction in IL-12 production may have an impact on DC antigen presenting function in frail elderly[231]. There is conflicting evidence regarding the function of antigen presenting cells with ageing[232]. Whilst some studies have shown a reduction in the ability of APCs from frail elderly to induce T cell proliferation, other studies have shown that DCs from the old do not have impaired capacity to induce T cell responses[233;234]. Castle et al found that APCs isolated from the peripheral blood of healthy old individuals had an enhanced ability to stimulate T cells from young individuals compared with APCs isolated from the young[235]. These differences may be due to selection of either healthy or old individuals, in addition to the subtype of APC used for the *in vitro* stimulation assay used.

To date, research on the effects of ageing on professional antigen presenting cells in the skin has focused almost exclusively on the Langerhans cell population. It is well recognized that the number of Langerhans cells in photoaged skin is reduced[236]. The clinical improvement in inflammatory skin diseases such as psoriasis following ultraviolet light exposure is thought, in part, to be related to depletion in the Langerhans cell population[237]. Change

in the number of Langerhans cells within sun-protected skin, however, remains controversial with some studies reporting a decrease in cell numbers [238;239]and others reporting no change with age[240]. The morphology of Langerhans cells is also thought to alter with ageing with a reduction in the number of dendrites and Birbeck granules[241].

Alterations in the function of Langerhans cells been reported. The migration of Langerhans cells in response to the intradermal injection of TNF-α has been shown to be reduced in the old[238] and this may be secondary to a reduction in IL-1β within the epidermis[242]. The capability of Langerhans cells to present antigens to sensitized T cells and to stimulate proliferation of allogeneic T cells has also been shown to be reduced in mice[243].

1.5.4 Effects of ageing on skin structure

Cutaneous ageing is a complex process and comprises both intrinsic ageing, thought to be genetically determined, and extrinsic ageing caused by environmental factors such as exposure to ultraviolet irradiation. Although there are microscopic differences between intrinsically and extrinsically aged skin, similar changes at a macroscopic level are seen with increased skin fragility, loss of elasticity, increased transparency, wrinkling and increased laxity[244]. These changes are thought to occur as a result of reduced proliferative capacity, cellular senescence and reduced biosynthetic capacity of skin-derived cells such as fibroblasts in addition to the increased production of degradative enzymes including the matrix metalloproteinases (MMPs)[245-247].

Collagen is most abundant extracellular component of the skin, accounting for 80% of dry weight, and provides the strong tensile properties of the dermis. An elastic fibre network provides elasticity accounts for 2-4% of extracellular matrix of sun-protected skin. Glycosaminoglycans/ proteoglycan macromolecules play a role in hydrating the skin and biological signalling. All three of these components are affected by ageing. In intrinsic skin ageing a reduction in the number and biosynthetic capacity of fibroblasts is observed

with an associated gradual loss of elastic tissue in the papillary dermis and a reduction in collagen content[246]. In extrinsic skin ageing there is an accumulation of elastotic material, with thickened, tangled granular amorphous structures thought to be degraded elastic fibres, and dysregulation of elastin and fibrillin production. This results in an associated gross distortion of the dermal matrix[246;248]. As with intrinsic ageing, a reduction in the collagen network is also seen.

1.5.5 Effects of ageing on cutaneous vasculature

The cutaneous vasculature is responsible for maintaining tissue viability, supplying oxygen and nutrition, and also allows for trafficking of cells in and out of the skin. The capillary network in the skin is made up of two main horizontal plexuses[249;250]: the superficial plexus just below the epidermis, where arterial capillaries rise to form the papillary capillary loops, and a deeper plexus at the junction between the dermis and subcutis. Ascending arterioles and descending venules connect the superficial and deep plexuses. The superficial capillary papillary loops are thought to be important for providing nutritional supply. In addition, perivascular infiltrates are seen in this site during DTH response. The deeper vessels are thought to contribute to the colour of skin and also thermoregulation[249].

Various non-invasive imaging techniques including videocapillaroscopy[251] and laser doppler flowmetry[252] have been employed to directly visualise the microcirculation in the skin. Patterns of blood flow in the microcirculation of the skin during the course of the Mantoux test have previously been studied using these techniques[253]. Maximal arteriolar vasodilatation was shown to occur during first 2 days of the reaction with rapid blood flow in the congested capillaries of the dermal papillae followed by engorgement of the deeper venous plexus in the resolving response at 72-96 hours[253].

Cutaneous disorders such as purpura[254], telangiectasia[255], palor[256], angioma and venous lake formation are seen more commonly in old individuals and indicate that age related changes in skin microvessels may occur[257]. The density of superficial capillary loops decreases with age in association with

atrophy of the superficial dermis, however overall blood flux has been shown to increase with an expansion of the parallel vasculature in the deeper dermis[250;258]. In one study, old individuals (60-74 years) were found to have a 66% reduction in capillary density on the volar (photoexposed) aspect of the forearm compared with the young group[250]. Similar findings have been demonstrated in mice[259]. However, marked variability in vasculature has been described according to the site studied and the degree of photoexposure[260;261].

Physiological changes in skin microvessels have also been observed including decreased vasoreactivity[261;262], decreased thickness of vessel basement membrane and decreased numbers of perivascular cells[263]. In addition, ageing has been shown to result in a decreased expression of eNOS, the constitutive isoform of nitric oxide (NO) synthetase, responsible for endothelium derived NO synthesis[264]. NO is thought to be a potent inhibitor of cellular adhesion, in particular monocytes and leukocytes, by the inhibition of the expression of adhesion molecules such as VCAM-1[265]. A reduction in endothelial nitric oxide production in the old has been proposed as a possible mechanism for development of atherosclerosis[264]. Other factors influencing vasodilatation may also be altered including sympathetic nerve responses[266] increased rigidity of the skin around blood vessels due to fibrosis[267] and impaired regulation of vascular calcium homeostasis/flux and sodium pump activity[266].

The effects of ageing on lymphatics in the skin are poorly understood although lymphatic involution with age within the main lymphatic trunks has been reported in addition to changes in the surrounding elastin fibre network in the skin that may increase rigidity with a resulting impairment of lymphatic drainage[268].

1.6 Models used to study immune responses in the skin

1.6.1 Differences between human and murine models.

Murine models are generally considered to be the mainstay of *in vivo* immunological experimentation as they allow for elegant manipulation of the immune system and in many ways mirror the human immune system. In addition, recent advances in light microscopy have allowed for direct *in vivo* visualization of evolving immune responses using intravital imaging in live animals[269]. Sequencing the human and mouse genome has shown remarkable conservation between the 2 species with only 300 genes that are unique to either species. Despite this, however, there are significant differences between mice and men in both the innate and adaptive immune systems[197;270] and so caution needs to be employed when extrapolating data from murine experiments to humans.

In the context of examining immune responses in the skin, DTH responses are assessed by the measurement of either footpad or ear swelling in mice compared with the forearm in humans. Previous experiments in humans have shown variation in the DTH response according to anatomical site[271;272] and therefore comparison of a forearm in a human and a footpad or ear in a mouse is difficult. In rats the resident T cells in the skin are also different with gamma delta TCR bearing T cells compared with alpha beta TCR in humans[273]. The duration of the response is altered in mice with a peak response at 12 hours compared with 2-3 days in humans[77]. This may be reflective of differences in antigen presentation and time required for antigen to be transported to the draining lymph nodes. Whereas the response consists primarily of lymphocytes in humans, it is neutrophil rich in mice[274]. Elicitation of the response also requires much higher concentrations of antigen in mice compared with humans[77].

The use of murine models to study ageing is also controversial[270]. Mice and men have dramatically different lifespans and although differences in T cell function have been found in both species this may be representative of different ageing mechanisms. Importantly, mice have significantly (10X) longer telomeres[275] and do not lose CD28 expression on T lymphocytes with

cellular differentiation[276], important for telomerase induction and therefore the restraints on immunity in humans due to telomere length may not be relevant in mice. Mice also have a reduced antioxidant defence and therefore may be more susceptible to the effects of oxidative stress with ageing compared with humans[192].

One of the difficulties with analyzing data from human studies is the presence of confounding factors due to poor standardization of the groups studied. The SENIEUR protocol was designed as a result of consensus form the EURAGE concerted action program on ageing[277] with a view to standardizing the population under study and excluding endogenous and exogenous influences on the immune system. The aim was to isolate pure ageing from that secondary to environment and disease. The SENIEUR protocol is based on the exclusion of disease based on clinical history, absence of biochemical abnormalities and absence of prescribed medication for known diseases or that with known influence on the immune system (including non-steroidal anti-inflammatory drugs). The use of the SENIEUR protocol in multiple studies has subsequently shown that it is a reliable instrument for the selection of volunteers, and that differences can be identified in immune parameters between the very healthy and the almost very healthy. In the very healthy, however, only very modest changes in the immune response have been identified up to an advanced age[131] and the clinical relevance of these changes has not been clarified.

However, it has been postulated that the SENIEUR protocol excludes over 90% of the aged population[278] and therefore is selecting a group of individuals poorly representative of the aged population as a whole. The study of individuals who are excluded by the SENIEUR protocol but well characterized in terms of clinical co-morbidity may be more relevant to the development of strategies to enhance quality of life with ageing. In this thesis, a modified version of the SENIEUR protocol was employed with a view to excluding significant comorbidity but selecting a representative group of old individuals.

1.7 Aims and objectives

It is known that there is a decline in immunity with age[191] that is thought in part to explain the observed increased frequency of infection and malignancy. Previous studies in old subjects, predominantly performed on nursing home residents, have indicated an increase in the percentage of negative DTH reactions in the skin to the Mantoux test and other antigens[160;161;279]. It has been assumed that this is reflective of a generalized decline in cell-mediated immunity with increasing age. However, it is unclear whether the previously reported decline in DTH responses in the old was related to associated co-morbidity or was a true reflection of reduction in their cutaneous immunity as a result of intrinsic ageing.

Previous studies have focused on clinical responses to the injection of antigen in the skin or cellular response in the peripheral blood, however it is not known how these parameters correlate with each other or if the skin is reflective of immunity in other compartments. Our laboratory has previously shown that there is dissociation between the peak clinical and peak cellular response in the skin during the course of a DTH response[64] and therefore the absence of a visible clinical response in the old may not be indicative of an absent immune response in the skin.

Given the demographic increase in age in the population it is essential that we have not only the tools to assess immunity but also have an understanding of the changes that occur in immunity with ageing.

AIM

To characterize the effects of ageing on the immune system in the skin in healthy old individuals.

OBJECTIVES

The objectives of this project were to:

1. Study the correlation between clinical DTH responses to the intradermal injection of antigen in the skin and peripheral blood responses to antigen in the old and young.
2. Characterize the lymphocytic response to the intradermal injection of antigen in the old.
3. Examine the skin homing capacity of memory T cells in the peripheral blood in the old.
4. Investigate the production of chemokines and cytokines at the site of the immune response in the old.
5. Evaluate cell types involved in the initiation of DTH responses in the skin.

2. Materials and Methods

2.1 Volunteer recruitment

Healthy young individuals under the age of 40 and old individuals over 70 were recruited for the study. Exclusion criteria based on a modified version of the Senieur protocol were used in order to prevent confounding factors due to co-morbidity and other factors that may influence the immune status of an individual (Table 2.1). All volunteers were Caucasian and living in the Greater London area. A total number of 53 old and 39 young individuals were recruited for the study. The age range for old volunteers was 70-91 years with a mean age of 79 years. The age range for young volunteers was 23- 37 years with a mean age of 28 years.

TABLE 2.1 (Exclusion criteria)

- Past history of TB
- Significant comorbidity (renal impairment or failure, heart failure, diabetes)
- Past history of inflammatory skin disorder
- Past history of neoplasia in the last 10 years
- Previous treatment with chemotherapy or radiotherapy
- Immunosuppressive medication
- Recent infection or immunization (within last month)
- Pregnancy and breast feeding
- Previous history of hypersensitivity to skin testing

Ethics

The study was approved by the Ethics committee of the Royal Free Hospital.

2.2 Skin testing

2.2.1 Mantoux tests

Mantoux test reactions were induced on the volar aspect of the forearm by the intradermal injection of 0.1ml of 10U/ml tuberculin purified derivative (PPD)

(Evans Vaccines Ltd., Liverpool, UK). Intradermal injection was confirmed by the induction of a distinct, sharply defined bleb at the skin test site. As a control, injection of the carrier solution for PPD (Evans Vaccines Ltd., Liverpool, UK) was also injected in the opposite forearm.

2.2.2 Candida skin testing

0.02ml of Candin skin test solution (Allermed Laboratories Inc, San Diego, CA) was injected intradermally as for the Mantoux test. As a control 0.02ml of sterile saline was injected, as the carrier solution for the Candin solution was not available commercially.

2.2.3 Varicella zoster virus (VZV) skin testing

0.02ml of VZV skin test solution (Gift from M. Takahashi, Osaka University) was injected as for the other antigens.

2.2.4 Assessment of skin test responses

Baseline skin erythema at the site of antigen injection was measured using a DermaSpectrometer (Cortex Technology, Hadsund, Denmark). This is a portable, handheld device that allows the measurement of the skin erythema index (EI) by measuring light absorption coefficients (Figure 2.1B). The mean of 3 measurements was recorded. At Day 3 and at the time of sampling (if different from Day 3) the change in EI from baseline, palpability and size of induration were measured. The change in EI was calculated by subtracting the baseline measurement from the EI taken when measuring the response. The size of induration was determined by measuring the maximum diameter of the response. The change in EI, induration and palpability were then scored according to Table 2.2 and the sum of the scores was combined to give an overall clinical score. Non-responding individuals were defined as having a clinical score of less than 1 at Day 3 and at any time point up to sampling.

TABLE 2.2 Assessment of skin test responses

Clinical Score	0	1	2	3	4	5	Score
Erythema-index (EI)	0	1-5	6-10	11-15	>16		
Size of induration (mm)	0	1-5	6-10	11-15	16-20	>21	
Palpability	Nil	Just palpable	Easily	Marked	Very marked		
						Total	

The change in EI from baseline, size of induration and palpability were scored and added together to give an overall clinical score.

2.3 Skin sampling

Skin suction blisters were raised or 5mm punch biopsies were taken from the site of skin testing. Samples were collected at various time points between 0 and 21 days after injection of antigen. Each volunteer was allotted to a specific sample time-point and either paired skin suction blisters and punch biopsies or blisters or biopsies alone were taken (Figures 2.1 and 2.2). Repeated skin testing to the same skin test antigen in the same individual was avoided in order to prevent boosting of the immune response from repeated antigenic exposure.

2.3.1 Suction blisters

Suction blister induction (Figure 2.2)

Skin suction has previously been shown to result in the formation of a split between the epidermis and dermis at the level of the lamina lucida. Suction blisters were induced by the application of negative pressure of 25-40kPa (200-300mmHg below atmospheric pressure) via a suction chamber (Medical Engineering, Royal Free Hospital, UK) centred over the site of testing for 2-4 hours using a clinical suction pump (VP25, Eschmann, Lancing, UK) (Figure 2.2). Skin suction chambers with apertures of 15mm, 12.5mm and 10mm were used according to the size of the response and skin elasticity. In all cases, the size of suction chamber aperture selected ensured that the whole of the area of induration was sucked up in to the chamber with minimal incorporation of normal surrounding skin.

Suction was applied at warm room temperature (~22°C) until a unilocular blister measuring 10-15mm was formed over the site of the skin test. The blister was then protected overnight with a rigid adhesive dressing assembled using a Comfeel plus ulcer dressing (Coloplast, Peterborough, UK), a universal top (Sterilin, Fisher Scientific UK Ltd, Loughborough, UK), Micropore tape (3M healthcare, Loughborough, UK) and Tubigrip bandaging (Seton Healthcare Group plc, Oldham, UK).

Blister fluid was aspirated from the blister at 18-24 hours after induction using a sterile 23G needle and a 2ml syringe (Tyco Healthcare UK Ltd, Gosport,

UK). The fluid was aspirated at the 18-24 hour time point in order to ensure maximal accumulation of cells within the blister fluid from the site of antigenic challenge in the skin. The recorded time of sampling was the time from skin injection to blister fluid aspiration. The volume of fluid recovered from the blister was recorded and suspended in 1.5ml conical tubes (Alpha Laboratories Ltd, Eastleigh, UK). The aspirated blister site was dressed with Betadine dry powder spray (Seton Healthcare Group plc, Oldham, UK) and a Mepore dressing (Molnlycke Health Care Ltd., Dunstable, UK). Volunteers were advised to leave the dressing in place and to keep it dry for 24 hours before removing it and leaving the wound open to the air. The suction cups were dismantled after use and disinfected in Barrycidal 36 (Heraeus Instruments Ltd, Brentwood, Essex, UK) for a minimum of 24 hours.

Suction blister cell isolation

The blister fluid was microcentrifuged at 650xg (3000 rpm) for 4 minutes (Microcentaur, MSE, Sanyo) to pellet the cellular contents. The supernatant was removed and aliquotted in to 1ml cryogenic tubes (Nunc, Thermofisher Scientific, Roskilde, Denmark) and stored at -70°C until analysed. The blister cell pellet was resuspended in 500µl of RPMI 1640(GIBCO, BRL Life Technologies, Paisley, UK) containing 10% human AB serum, 100U/ml penicillin, 100µg/ml streptomycin and 2mM L-glutamine (all from Sigma Aldrich, Gillingham, Dorset, UK).

Counting of blister cells

Blister white and red cell numbers were quantified using a haemocytometer. Cellular viability was assessed by trypan blue exclusion. A 10µl aliquot of blister cell suspension was mixed 1:1 with Trypan blue (Sigma- Aldrich, Gillingham, Dorset, UK) and viable non-stained cells were counted.

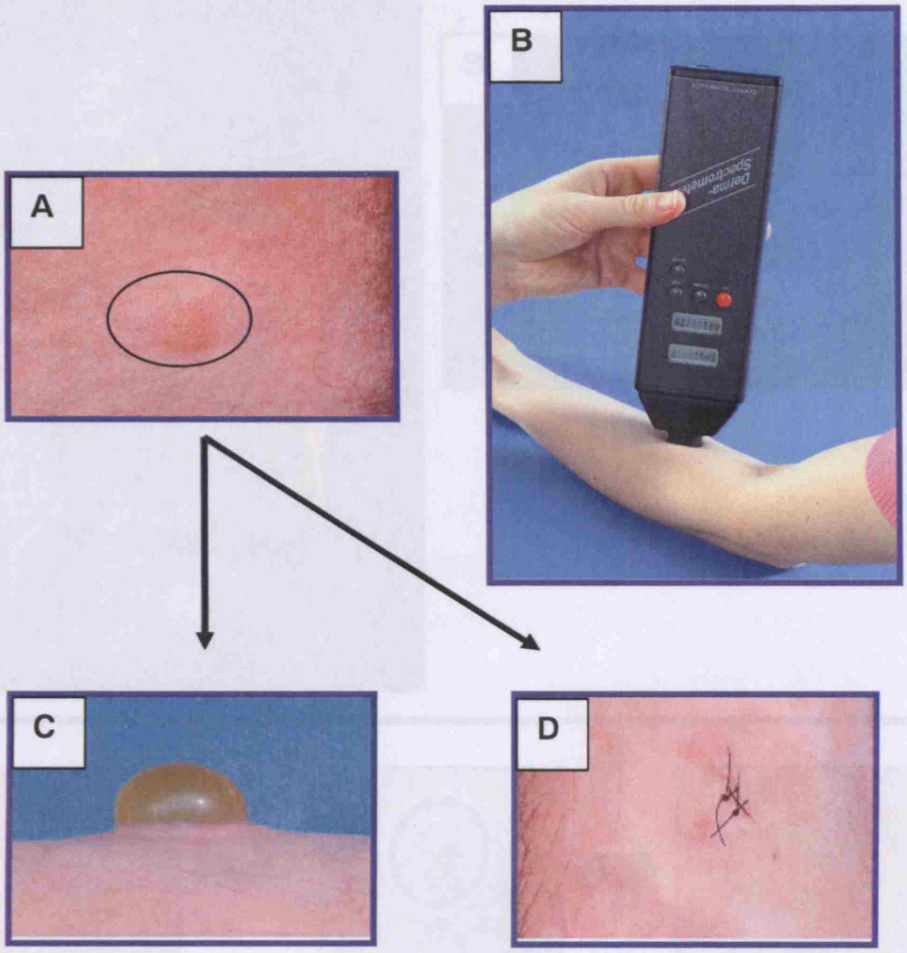


FIGURE 2.1 The clinical DTH response in the skin and skin sampling.

A. The skin test response to the intradermal injection of antigen is characterized by the development of erythema and induration at the site of injection, reaching a peak at 2-3 days.

B. The erythema index at the site of the response was measured at baseline, at day 3 and at the time of sampling using a DermaSpectrometer. The mean of 3 measurements was recorded.

C. Following skin testing on the inner aspect of the forearm, skin samples were collected at various time points from 0-15 days after injection. Skin suction blisters were raised primarily to obtain either cutaneous lymphocytes or monocytes.

D. Skin biopsies were also taken. Whenever possible paired biopsies and blisters were taken from opposite forearms. Venous blood was also taken for PBMC isolation.

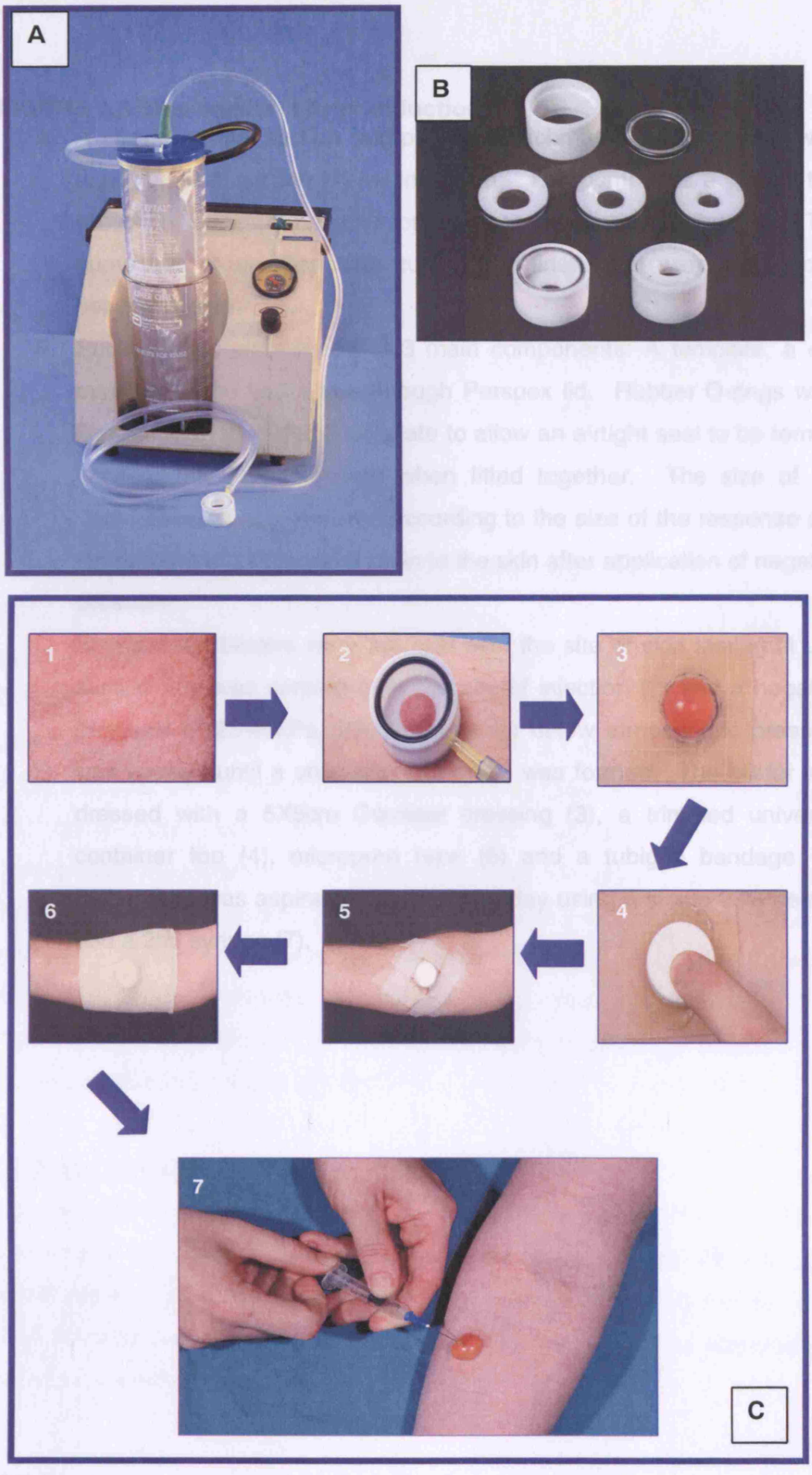


FIGURE 2.2

FIGURE 2.2 Skin suction blister induction.

A. A clinical grade suction pump (VP25 Eschmann suction pump) was used for skin suction blister induction. The pump has a guage that allows for constant negative pressure to be applied to the skin. The pump has sterile disposible tubing and liners that were changed in between volunteers.

B. Suction cups were made of 3 main components: A template, a cup made of nylon and a see through Perspex lid. Rubber O-rings were fitted around the lid and template to allow an airtight seal to be formed between all 3 components when fitted together. The size of the template used was adjusted according to the size of the response and the amount of skin sucked up in to the skin after application of negative pressure.

C. Skin suction blisters were induced over the site of skin testing (1). A suction cup was centred over the site of injection (2) and a negative pressure of 25-40kPa (200-300 mmHg) below atmospheric pressure was applied until a unilocular blister (3) was formed. The blister was dressed with a 5X5cm Comfeel dressing (3), a trimmed universal container top (4), micropore tape (5) and a tubigrip bandage (6). Blister fluid was aspirated the following day using a sterile 23G needle and a 2ml syringe (7).

2.3.2 Skin biopsy

Biopsy procedure

A 5mm punch biopsy was taken either from the centre of the site of injection of antigen or control solution or normal skin (Figure 2.1D). The surrounding skin was infiltrated with 2% lignocaine/ 1:80, 000 adrenaline local anaesthetic (Astra Pharmaceuticals Ltd, Kings Langley, UK) prior to biopsy. The wound was closed with 4/0 Surgipro polypropylene suture (Tyco Healthcare UK Ltd., Gosport, UK). The skin was transported as quickly as possible to the lab in sterile saline.

Biopsy storage and sectioning

Biopsies were mounted in Cryo-M-Bed (Bright Instrument Company Ltd., Huntingdon, UK) on cork disks, orientated so that the epidermis was perpendicular to the cork disk, and snap frozen in isopentane (Sigma-Aldrich, Gillingham, Dorset, UK) cooled in a bath of liquid nitrogen. The samples were then stored in a freezer at -80°C. 6μm frozen sections were cut at -20°C using a Bright 5040 microtome (Bright Instrument Company Ltd., Huntingdon, UK) on to poly-L-lysine coated glass slides (Sigma-Aldrich, Gillingham, Dorset, UK). Poly-L-lysine coated slides were used in order to promote strong adhesion of the skin section to the slide. Two sections were mounted on to each slide. The sections were then left overnight to air-dry and then fixed in fresh acetone for 10 minutes, followed by 99% Ethanol for 10 minutes. The sections were air-dried for 10 minutes and then cling-filmed wrapped and stored until use in a freezer at -80°C.

2.4 Blood samples

Blood was taken from all volunteers prior to skin testing and at the time of skin sampling for the isolation of peripheral blood mononuclear cells (PBMCs) and serum separation. The serum sample was refrigerated for 30 minutes and then centrifuged at 800xg for 20 minutes. The supernatant was aliquotted in to 1ml cryogenic tubes and stored at -80°C.

2.4.1 PBMC isolation

Heparinised blood was mixed 1:1 with Hanks Balanced Salt Solution (HBSS) (GIBCO, BRL Life Technologies, Paisley, UK) and layered on to Ficoll-Paque (Amersham Biosciences UK Ltd, Chalfont St. Giles, UK) in 50ml Falcon tubes. This was centrifuged for 20 minutes at 800xg with no brake. The buffy coat at the interphase layer was harvested and then washed twice for 10 minutes in excess HBSS by centrifugation at 650xg for the first wash and 300xg for the second wash. The cells were finally resuspended in complete medium as for the blister cells (RPMI 1640 (GIBCO, BRL Life Technologies, Paisley, UK) containing 10% human AB serum, 100U/ml penicillin, 100μg/ml streptomycin and 2mM L-glutamine (all from Sigma Aldrich, Gillingham, Dorset, UK)). Typically, 1-2 $x10^6$ cells were isolated per 1ml of venous blood.

2.5 Flow cytometry

Flow cytometry was used in order to identify and analyse specific subsets of cells. The technique allows for the rapid measurement of individual cells as they flow in a fluid stream one by one through a laser beam. Cells are analysed on the basis of cell size and granularity in addition to labeling with antibodies conjugated to different fluorochromes. A FACSCalibur flow cytometer (Becton Dickinson, Oxford, UK) was used for all experiments. This machine has 2 lasers (488nm and 600-650nm), enabling the simultaneous measurement of 4 different coloured fluorochromes (channels FL1-FL-4). Acquisition and analysis of all data was undertaken using CellQuest software (Becton Dickinson, Oxford, UK). Single colour fluorochrome controls were used prior to the start of sample acquisition in order to compensate between different channels to correct for overlapping emission spectra that can occur between different fluorochromes. The optimum concentration of each antibody was determined by preliminary titration.

FLOW CYTOMETRY				
Staining	Specificity/ fluorochrome	Clone	Isotype/source	Company
Direct/surface				
	CXCR4 PE	12G5	Mouse antihuman IgG2a	R&D Systems
	CCR8 PE	191704	Rat antihuman IgG2b	R&D Systems
	CXCR3 (CD183) APC	1C6/CXCR3	Mouse antihuman IgG1k	BD Pharmingen
	CCR4 PE	1G1	Mouse antihuman IgG1k	BD Pharmingen
	CLA FITC	HECA-452	Rat IgM k	BD Pharmingen
	CD11a	HI111	Mouse antihuman IgG1k	BD Pharmingen
	CD4 PerCP	SK3	Mouse antihuman IgG+IgG1k	BD Pharmingen
	CD4 PECy5.5			Caltag
	CD3 APC	UCHT-1	Mouse antihuman IgG1k	Dakocytomation
	CD69 PE	L78	Mouse antihuman IgG1k	BD Pharmingen
	CD27 PE	M-T271	Mouse antihuman IgG1k	BD Pharmingen
	CD28 FITC	CD28.2	Mouse antihuman IgG1k	BD Pharmingen
Direct/ intracellular				
	Ki67 FITC	B56	Mouse antihuman IgG1	BD Pharmingen
	Ki67 isotype control	CTL-MOPC-21		
	IFN-gamma APC	B27	Mouse antihuman IgG1	BD Pharmingen

All antibodies shown are monoclonal.

FITC: Fluoroscein isothiocyanate

PE: Phycoerythrin

PerCP: Peridinin clorophyll protein

PECy5.5: Phycoerythrin- cyanin 5.5

APC: Allophycocyanin

TABLE 2.3 Antibodies used for flow cytometry.

Analysis was performed by gating on either the live cell (lymphocyte or monocyte) populations using forward and side scatter profiles with exclusion of dead cells and debris. Isotype and negative controls were used to delineate positive populations where positive and negative populations were not clearly distinguishable. The antibodies used for flow cytometry are shown in Table 2.3.

2.5.1 Surface staining by direct immunofluorescence

The expression of cell surface markers was determined using monoclonal antibodies directly conjugated to a specific fluorochrome with a single incubation step. The use of a combination of different fluorochromes such as FITC, PE, PerCP and APC allowed for the detection of up to 4 different antigens simultaneously. 100µl of blister or PBMC cell suspensions in culture medium were aliquotted in to 5ml FACS tubes (Falcon, Becton Dickinson Labware, New Jersey, USA) together with pre-determined optimal concentrations of directly conjugated antibodies. The tubes were vortexed and incubated in the dark at room temperature for 15 minutes. The cells were then washed in excess PBSA (1% w/v bovine serum albumin and 0.02% sodium azide (both Sigma- Aldrich, Gillingham, Dorset, UK) in phosphate buffered saline (PBS)) and centrifuged at 650xg for 5 minutes. The supernatant was decanted and the cells resuspended and fixed with 200 µl of 2% paraformaldehyde (Sigma- Aldrich, Gillingham, Dorset, UK) in PBS and stored for at least 1 hour in the dark at 4°C until analysed.

2.5.2 Intracellular staining

In order to stain for intracellular antigens such as Ki67 or cytokines, cells were fixed and permeabilised to allow for the access of antibodies to intracellular structures whilst maintaining the morphological scatter characteristics of the cells. A modified protocol of the Caltag Fix and Perm Cell Permeabilisation kit (Caltag Laboratories, Burlingame, California, USA) was used. If a combination of both cell surface and intracellular antigen staining was required, cells were first incubated with the cell surface antigen antibodies as per protocol above, washed with PBSA and centrifuged at 650xg for 5 minutes. The supernatant

was decanted and the cell pellet was resuspended with 100µl of reagent A (Fixation medium containing formaldehyde). The tubes were vortexed and left to incubate in the dark at room temperature for 10 minutes. The cells were washed with PBSA and centrifuged at 650xg for 5 minutes. The cell pellet was then resuspended with 100µl of reagent B (Permeabilisation medium) and optimal concentrations of directly conjugated antibodies to intracellular antibodies were added. The tubes were vortexed and left to incubate in the dark at room temperature for 15 minutes. Finally the cells were washed with PBSA, centrifuged at 650xg for 5 minutes and fixed with 2% paraformaldehyde as described for the direct immunofluorescence method.

2.5.3 Detection of antigen specific cells

Antigen specific CD4+ lymphocytes were identified using flow cytometry to detect the intracellular accumulation of interferon gamma following short term *in vitro* stimulation with antigen in the presence of brefeldin A. Brefeldin A is a fungal metabolite that enhances the staining of intracellular cytokines, preventing their secretion by interfering with their transport in vesicles from the rough endoplasmic reticulum to the Golgi complex. PBMCs ($1x10^6$) or blister cells in complete medium were added to sterile FACS polypropylene tunes (Kendall, Tyco Healthcare Group, Massachusetts, USA). The cells were stimulated with PPD (Statens serum institute, Copenhagen, Denmark) at a final concentration of 10µg/ml for 15 hours at 37°C in a humidified 5% CO_2 atmosphere. Brefeldin A (Sigma- Aldrich, Gillingham, Dorset, UK) was added at a final concentration of 5µg/ml after 2 hours of incubation. The same protocol was used to detect CMV antigen specific cells with the substitution of PPD antigen for CMV lysate (Virusys Corporation, Sykesville, MD, USA) at a final concentration of 100µl/ml.

A modified protocol, with prolonged incubation prior to the addition of brefeldin A, was used to detect lymphocytes specific for Candida and VZV antigens in order to maximize the detection of interferon gamma positive cells. PBMCs and blister cells were incubated with Candida antigen (Greer laboratories, Nuway Circle, Lenoir, USA) at a final concentration of 40µg/ml and VZV

antigen (Virusys corporation Sykesville, MD, USA) at a final concentration of 20μl/ml for 15 hours followed by the addition of brefeldin A at 15 hours and then staining for interferon gamma at 20 hours. For experiments where CLA expression on antigen specific cells was analysed, unsupplemented X-Vivo medium (Biowhittaker, Cambrex Bioscience, Walkersville.) was used rather than complete medium to culture the cells as this was found to prevent the down-regulation of CLA upon cell stimulation.

For both protocols unstimulated PBMC and blister cell controls were used to verify the staining specificity and as a guide for delineating positive and negative populations. As a positive control, the superantigen Staphylococcal exotoxin B (SEB) was added to PBMCs and blister cells at a final concentration of 1μg/ml (Sigma- Aldrich, Gillingham, Dorset, UK). Gating was set on the live lymphocyte population using forward and side scatter profiles.

2.5.4 Enumeration of T lymphocytes

Absolute cell counts of T lymphocytes in PBMCs and blister cells were performed using TruCOUNT^{TM} tubes (BD Biosciences, San Jose, California, USA) and flow cytometric analysis. This technique was used to determine the absolute number of cells extracted from the skin following suction blister induction and the number of cells that had migrated in the transwell migration assay experiments. A known volume of cell suspension was added to a known number of fluorescent beads contained within the TruCOUNT tube. During sampling the absolute number of cells in the sample was determined by comparing the number of acquired gated cellular events and the gated fluorescent bead events. Cells added to the tube were also labeled with fluorescent surface markers in order to calculate the absolute number of different types of cells.

100μl of the PBMC or blister cell samples in complete culture medium was carefully pipetted in to a TruCOUNT tube to which the required antibodies to surface antigens were added at concentrations as for direct immunofluorescence staining. The tubes were vortexed and incubated in the

dark at room temperature for 15 minutes. After, incubation 200µl of 1X FACS lysis buffer (BD Biosciences, San Jose, California, USA) was added and the tube was vortexed. The sample was stored for a minimum of 1 hour in the dark at 4°C prior to acquisition and analysis. The absolute number of cells was calculated using the equation below:

$$\frac{X \text{ gated cell events} \times Y \text{ beads per TruCOUNT tube} \times \text{sample volume}}{Z \text{ gated bead events} \times \text{Trucount volume}}$$

2.6 *In vitro* cell culture

2.6.1 Measurement of cellular proliferation by [^{3}H] thymidine incorporation

PBMCs were suspended in complete culture medium (RPMI (GIBCO, BRL Life Technologies, Paisley, UK) containing 10% human serum supplemented with 100U/ml penicillin, 100µg/ml streptomycin and 2mM L-glutamine (all from Sigma- Aldrich, Gillingham, Dorset, UK)). The PBMC concentration used was $1x10^6$ cells/ml. 100µl aliquots of cell suspension were pipetted in to the wells of 96 well round bottomed cell culture plates (Falcon, Becton Dickinson Labware, New Jersey, USA) and incubated at 37°C in a humidified 5% CO_2 atmosphere. 100µl of antigen diluted in complete medium was added in a range of concentrations to wells, with each concentration performed in triplicate. The range of final well antigen concentrations were as follows: PPD antigen (Statens serum institute, Copenhagen, Denmark) 0-5µg/ml, candida antigen (Greer laboratories, Lenoir, NC, USA) 0-20µg/ml and VZV antigen (Virusys corporation, Sykesville, MD, USA) 0-20µl/ml. The cells were incubated for 5-6 days (5 days for PPD antigen, 6 days for candida and VZV antigens) before adding 10µl of 0.0025MBq [^{3}H] thymidine (Amersham Biosciences UK Ltd, Chalfont St. Giles, UK) to each well and incubating for an additional 16 hours. Optimal incubation times were determined by initial visual assessment of wells for signs of cell proliferation and then measurement of proliferation by [^{3}H] thymidine incorporation at different time points. Cells were harvested onto glass microfibre filter strips using a cell harvester (Cambridge Technology, Watertown, Massachusetts, USA) and

counts per minute (cpm) from incorporated [^{3}H] thymidine were determined by liquid scintillation counting. The mean of triplicate wells was calculated and used for the purpose of data analysis.

2.7 Cytokine/ chemokine assays

2.7.1 Multiple cytokine assay by cytometric bead array (BD™ Cytometric Bead Array (CBA) Cell Signalling Flex Set System)

Concentrations of cytokines and chemokines (IL-6, IL-8, IL-10, TNF, IFN-γ, RANTES, MCP-1, MIP-1α) in blister fluid and serum were analysed using the BD™ Cytometric Bead Array (CBA) Cell Signalling Flex Set System and were analysed using a BD FACSArray™ bioanalyzer with FCAP Array Software (all BD Biosciences, San Jose, California, USA). This technique employs the use of particles with discrete fluorescent intensities and sizes to detect soluble analytes and enables multiplexed quantitative analysis of multiple proteins from a single sample using flow cytometry.

The assay was performed in a 96 well round bottomed plate (Falcon, Becton Dickinson Labware, New Jersey, USA). 50μl of mixed capture beads each coated with a cytokine- specific antibody was mixed with 50μl of mixed cytokine-specific PE-conjugated antibodies in addition to 50μl of blister fluid, serum or dilution series of analyte recombinant protein. The plate was gently vortexed and then incubated at 4 hours in the dark, at room temperature, in order for sandwich complexes to form. The plate was then centrifuged and the supernatant aspirated from each well. 150μl of wash buffer was then added to each well and the plate was then inserted in to the BD FACSArray™ bioanalyzer for analysis. This machine automatically assigns an alpha-numeric position on a grid for each bead population using the NIR and Red channels. A standard curve for each analyte is created based on bead size and PE- fluorescence intensity for the dilution series of the recombinant protein samples. This enables the quantitative calculation of the concentration of the analytes from the test samples bound to the beads.

All samples were analysed in one experiment in order to minimize variation in measured concentrations due to different experimental conditions on different days. In addition, blister and serum samples were stored at -70°C immediately after collection from volunteers and were only defrosted for the purpose of analysis in order to minimize changes in cytokine/chemokine concentration due to repeated freeze thaw cycles.

2.7.2 Multiplex bead immunoassay (Luminex technique)

Analysis of the chemokines CCL27 (CTACK), CCL17 (TARC), CCL1 (I-309) and SDF was performed by Dr. John Curnow (Division of Immunity and Inflammation, MRC Centre for Immune Regulation, University of Birmingham, UK.). Cytokine Beadlyte and Luminex 100 assay kits were used (both from Upstate Biotechnology, Lake Placid, New York, USA.). The technique is very similar to that described for the other cytokine/chemokine assays and was selected for the analysis of some chemokine concentrations due to the lack of availability of reagents using the BD™ Cytometric Bead Array (CBA) Cell Signalling Flex Set System.

The technique is based on the use of sets of polystyrene microspheres, each internally dyed with ratios of two spectrally distinct fluorochromes that are distinct for each set of microsphere. Each set of microspheres is conjugated with a specific cytokine capture antibody. A biotinylated antibody that binds to streptavidin labeled with a third fluorochrome binds to captured chemokines, present on the surface of the microspheres. The Luminex 100 flow cytometer and software is able to calculate the concentration of chemokines by separating pools of microspheres with different specificities in to individual bead sets and by determining the amount of the analyte bound to the surface of each microsphere.

2.7.3 Transwell chemotaxis assays.

In vitro cell migration assays were used to quantify the ability of CLA+ lymphocytes to migrate in response to selected chemokines. Cell migration in response to IP-10 (I-309) and SDF recombinant peptides (both Peprotech Inc,

New Jersey, USA) was measured. SDF was used as a positive control as large numbers of cells have previously been shown to migrate in response to this chemokine *in vitro*. 400µl of complete medium with 500ng/ml of either recombinant IP-10 or SDF was added per well in to 26 well flat- bottomed plates. 6.5-mm diameter, 3-µm pore transwell inserts (Greiner Bio-one Ltd, Stonehouse, UK) were placed in to each well. As a negative control complete medium alone was added in to wells. 200µl of PBMC suspension in complete medium at a concentration of $2.5x10^6$ cells/ml was pipetted in to the top chamber. Triplicate wells were used for each volunteer and chemokine. The plates were incubated for 3 h at 37°C in a humidified 5% CO_2 atmosphere. Cells were then collected in to FACS polypropylene tubes (Kendall, Tyco Healthcare Group, Massachusetts, USA) from both top and bottom chambers. All chambers were rinsed twice with complete medium in order to maximize cell retrieval. The cells were then centrifuged at 650xg for 5 minutes, the supernatant was discarded and the pellet was re-suspended in 100µl of complete culture medium. This was transferred to a TruCOUNT™ tube, stained with anti-CLA, CD3 and CD4 antibodies, acquired and analysed according to the protocol described in section.

HISTOLOGY				
Indirect immunoperoxidase				
(HRP- Polyclonal rabbit antimouse-	CD1a	NA1/34	Mouse antihuman IgG2ak	Dakocytomation
Dakocytomation)	Ki67	Ki-67	Mouse antihuman IgG1k	Dakocytomation
4µl per slide	CD3	UCHT-1	Mouse antihuman IgG1k	Dakocytomation
	CD68	EBM11	Mouse antihuman IgG1k	Dakocytomation
	CD8	DK25	Mouse antihuman IgG1k	Dakocytomation
	CD14	TUK4	Mouse antihuman IgG2ak	Dakocytomation
	CD4 (Pure Leu-3a)		Mouse antihuman IgG1k	BD Pharmingen
Indirect immunoperoxidase	HLA-DR (biotin)			Immunotech
(Streptomycin-HRP kit BD)	DC-SIGN (CD209) biotin	DCN46	IgG2b	BD Pharmingen
Negative controls	IgG2b			Dakocytomation
	IgG2a	DAK-G05		Dakocytomation
	IgG1k	107.3		BD Pharmingen
	IgG2ak			BD Pharmingen

TABLE 2.4 Antibodies used for immunohistochemistry

2.8 Immunohistology

The study employed the use of both indirect immunoperoxidase and biotin/streptavidin alkaline phosphatase techniques. The characteristics of the monoclonal antibodies used are documented in Table 2.4.

2.8.1 Indirect Immunoperoxidase technique

The indirect immunoperoxidase technique was used to detect numbers and distribution of T cells, monocytes/ macrophages, dendritic cells and to detect cellular proliferation in the skin biopsies. In this technique a primary unconjugated antibody is bound to the antigen of interest in the tissue section. A second horseradish-peroxidase conjugated antibody, raised in another animal host that is specific for the animal and immunoglobulin class of the primary antibody, is then bound to the primary antibody. The complex that is formed is visualized following incubation with an appropriate chromagen or substrate. In this study, the chromagen 3, 3'-diaminobenzidine tetrahydrochloride (DAB) was used. This is converted by horseradish-peroxidase into an insoluble brown substrate, identifying the cell/ antigen of choice.

Frozen tissue sections were defrosted and placed in to a moist staining chamber containing water soaked tissue. The sections were ringed with polysiloxane (Dakocytomation, Glostrup, Denmark) as a water repellent. 50µl of 1x phosphate buffered saline (PBS) (GIBCO, Invitrogen, Paisley, UK) containing 10% human serum (Sigma Aldrich, Gillingham, Dorset, UK), 10% Aprotinin (Sigma- Aldrich, Gillingham, Dorset UK) and a pre-titrated concentration of primary antibody was carefully pipetted on to each slide. The antibodies used are listed in Table 2.4. Non-specific antibody binding was minimized by the addition of 10% human serum. 10% Aprotinin (a protease inhibitor derived from bovine lung) was added to block any endogenous immunoperoxidase activity within the tissue. The slides were then left to incubate for 45 minutes at room temperature. The fluid on the sections was removed by gently tapping the slide and the sections were then washed with fresh 1xPBS for 5 minutes, followed by formalin buffered saline (4.5g sodium dihydrate phosphate, 6.5g di-sodium hydrogen orthophosphate, 100ml of 40% formaldehyde and 900ml of 1x PBS) for 10 minutes and finally 1xPBS for 5 minutes. 4µl of horseradish peroxidase conjugated rabbit anti-mouse secondary antibody in 50µl of 1xPBS with 10% human serum was then added to each section. The sections were incubated at room temperature for a further 45 minutes and then washed as previously.

1 drop of Filtered Nickel DAB solution (95 mg of 3.3'diaminobenzidine, 1.6g NaCl, 0.136g Imidazole, 2g nickel sulfate dissolved in 180ml of Tris/HCL buffer with pH corrected to 7.4 with 1M TRIS) containing 1µl/ml of 10% H_2O_2 (added immediately prior to use) was then added to each section using a Pasteur pipette. This was left for up to 7 minutes prior to rinsing with PBS for 3 minutes. Slides were then placed in Tris Cobalt to intensify the stain (1.2g TRIS base, 1g Cobalt Chloride dissolved in 180ml distilled H_2O with pH corrected to 7.2 with HCl) for 5 minutes and then rinsed in tap water. Sections were counterstained with 0.1% Nuclear fast red for 5 minutes, rinsed in tap water and then dehydrated by washing for 10 seconds in 70% ethanol, 90% ethanol, twice in neat ethanol and then cleared in 50:50 Citroclear/ ethanol mix and finally neat Citroclear (HD Supplies, Aylesbury, Bucks, UK). Sections

were left in the neat Citroclear for a further 10 minutes and then mounted in styrolite.

2.8.2 Control slides

Three control preparations were used each time staining was performed. As a negative control, a section of both tonsil and skin tissue was incubated with normal mouse serum instead of primary antibody. In addition, where possible, a negative isotype control antibody for the same animal species as the primary antibody was also used at the same concentration on single sections of skin and tonsil (Table 2.4). Tonsil tissue was also stained as a positive control. Tonsil provides a rich source of cells including those stained for in the skin sections and allows for the distribution and staining of cells to be tested against tissue architecture.

2.8.3 Biotin/streptavidin/horseradish peroxidase technique

An indirect biotin/ streptavidin method was used to detect DC-SIGN expression on skin tissue sections. In this technique, the primary antibody is allowed to bind with specific antigen in the tissue sections. A secondary biotinylated antibody is then applied. This is raised in a different species to the primary antibody and is specific for the primary antibody species and immunoglobulin class. A third layer consisting of streptavidin conjugated to horseradish peroxidase is then added. Streptavidin has a strong affinity for biotin. Up to 150 biotin molecules can be complexed to a single second layer antibody, allowing for amplification of the staining signal and a greater sensitivity than the indirect immunoperoxidase technique. As for the indirect immunoperoxidase technique, DAB is then added which acts as a substrate for horseradish peroxidase.

Frozen sections were placed in to 1x PBS for 2 minutes. The sections were then ringed with polysiloxane and incubated with 50µl of 1x PBS containing 10% human serum, 10% aprotinin and a pre-tritrated concentration of primary mouse antihuman DC-SIGN antibody. The sections were incubated for 1 hour at room temperature in a moist chamber with a lid and then washed in 1x PBS

for 5 minutes, formalin buffered saline (made up as described previously) for 10 minutes and then 1xPBS for 5 minutes. The sections were incubated with 50µl of 1xPBS with 10% human serum and secondary biotinylated antibody (description and supplier) for 30 minutes and then washed as previously. The DAB, counterstaining, dehydration, clearing and mounting steps were performed in precisely the same way as for the indirect immunoperoxidase technique.

Staining for HLA-DR on sections was performed using a primary pre-biotinylated mouse anti-human antibody to HLA-DR. The protocol was therefore amended for this antigen, with omission of the addition of the secondary biotinylated antibody. The protocol was otherwise identical to that stated above.

2.8.4 Quantification of Immunohistochemistry

A single observer using coded slides carried out all measurements. The slides were visualized using a light microscope (Nikon Eclipse E600) and a digital camera (Nikon DXM 1200F camera with Eclipse Net software version 1.16.3 for Nikon). High-resolution colour images taken of the required fields were saved in tif (uncompressed file) format and then analysed using Image J software (downloaded from NIH website). In all cases, a frame with a predetermined area of 15 X 10cm was centred on the digital image and the number of cells within it was counted by clicking on the image with a mouse linked to the computer. For each section, 5 representative fields were selected for analysis and the mean value of the cell count was recorded. When counting the numbers of cells in perivascular infiltrates, the 5 largest perivascular infiltrates present in the upper and mid dermis were selected for analysis. Cell numbers were expressed as the absolute number of cells counted within the frame. For the assessment of Langerhans cell numbers the cell number was expressed as cells per epidermal unit area. The area within which cells were counted was measured by drawing around the outline of the epidermis and then analysed using Image J software.

2.9 Statistics

Statistical analysis was performed using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, California, USA). Non-parametric tests were predominantly utilised as the data could not be assumed to be normally distributed. The Kruskall-Wallis test was used to compare three or more unpaired groups and the Mann- Whitney was used when comparing only two unpaired groups. The Wilcoxon matched pairs test was used when comparing two groups of matched data. Asterixes are shown on some graph figures to indicate the p value and are rated as follows: $p<0.05$ *, $p<0.01$** and $p<0.001$***.

3. Cutaneous and peripheral blood responses to antigen in the young and old.

3.1 Introduction

The delayed-type hypersensitivity response to the intradermal injection of antigen is traditionally thought to be a memory T cell mediated response, representative of underlying cell-mediated systemic immunity. DTH testing in the skin therefore potentially provides a useful clinical technique for assessing an individual's immunity to different infections. The cutaneous response to the injection of tuberculin purified protein derivative (PPD), known as the Mantoux test (MT), is the standard method for determining immunity to tuberculosis (TB) and screening individuals for latent TB (NICE guidelines, March 2006). It is also used widely in research as the prototypic model for delayed type hypersensitivity responses (DTH) in the skin.

In humans, a positive clinical response to the injection of PPD antigen in to the skin is typically characterised by erythema and swelling that peaks at 48-72 hours and resolves within 14 days. Previous studies in old subjects, predominantly performed on nursing home residents, have indicated an increase in the percentage of negative DTH reactions to the Mantoux test (MT) and other antigens[160;161;279]. It has been assumed that this is reflective of a generalised decline in cell- mediated immunity with increasing age. However, individuals of all ages with diseases such as rheumatoid arthritis[280], chronic renal failure[281], and malnutrition[282] have also been observed to have a decreased cutaneous responsiveness to the MT. It is therefore unclear whether the previously reported decline in DTH responses in the old is related to associated co-morbidity or is a true reflection of reduction in their immunity as a result of intrinsic ageing.

Previous studies have focused on clinical responses to the injection of antigen in the skin or cellular responses to antigen in the peripheral blood, however it

is not known how these parameters correlate with each other or if the skin is reflective of immunity in other compartments. Responses to a variety of antigens both in the peripheral blood and skin of old and young individuals were therefore investigated in order to assess whether DTH skin test responses are truly representative of global immunity to infection.

3.2 The Mantoux test

3.2.1 Clinical responses to the Mantoux test

A total of 35 old and 20 young individuals were tested with the intradermal injection of 1 unit of PPD (0.1ml of a 1:10000 solution of PPD skin test solution) into the inner aspect of the upper forearm. In previous studies both 10 and 1 unit doses of PPD have been used in order to elicit DTH responses. It has been shown, however, that no difference in clinical response was seen between the 2 doses in young individuals who respond after the first test with PPD (Unpublished data, Reed JR). The lower dose of PPD was therefore chosen for the purpose of this study in order to more readily identify any potential differences between the young and old groups.

In all individuals who developed a response, the peak clinical response occurred between 48 and 72 hours as demonstrated previously (Figure 2.1). At 72 hours the diameter of the response, palpability and change in erythema from baseline in the skin were measured and a clinical score was assigned (Table 2.2). Previously, the clinical score has been found to parallel the degree of erythema and the diameter of the lesion (Unpublished data, Cate Orteu). The inclusion of a measurement of palpability (induration) in the clinical score was added as this was thought to be a more representative reflection of the degree of inflammation in the lesions. In this study, the degree of induration was also found to correlate with the clinical score in both the young and old groups.

The sum of the clinical scores for the old and young groups was used to calculate a mean score for both groups. Overall there was a significantly reduced clinical score ($P<0.0001$ Mann Whitney test) in the old group (Mean: 0.19, Median: 0) compared with the young (Mean: 6.4, Median: 7.5) (Figure

3.1). Only 4 old individuals (11% of old people tested) developed a detectable clinical response to the MT. The peak and duration of the clinical response in these 4 old individuals did not differ from the clinical response in the young group.

FIGURE 3.1 Clinical response to the Mantoux test in the young and old. The clinical response to the intradermal injection of 1 unit of PPD was assessed in the young and old at Day 3. This showed a significant reduction in clinical score (Mann Whitney test $p<0.0001$).

3.2.2 Peripheral blood responses to the Mantoux test

We next investigated whether the lack of response in the skin reflected a global loss of reactivity to the PPD antigen. The ability of peripheral blood mononuclear cells to proliferate in response to antigen is thought to be reflective of an individual's ability to mount a response to systemic infection. We therefore examined the functional ability of PBMCs isolated from the blood to respond to the PPD antigen using proliferation assays. Purified PBMCs were stimulated with a range of doses of PPD antigen from 0-10μg/ml and proliferation was measured by tritiated thymidine incorporation at Day 5. *In vitro* PBMC proliferation assays with PPD showed no statistical difference in

the ability of cells to proliferate in the young and old groups at concentrations of antigen at both 1μg/ml (Mann Whitney p=0.105) and 10μg/ml (Mann Whitney p=0.514) (Figure 3.2)

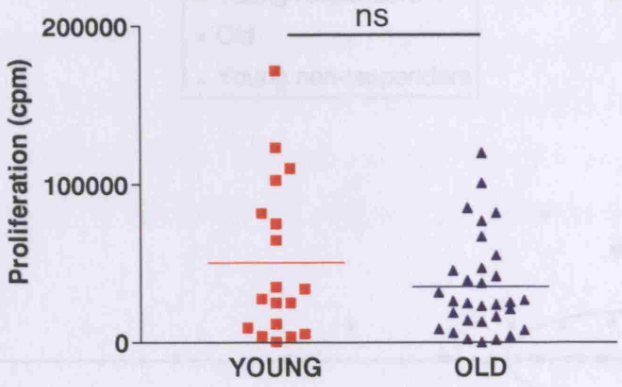


FIGURE 3.2 PBMC proliferation in response to PPD antigen (10μg/ml) in the young and old.

Following 5 days of culture with PPD, the ability of young and old PBMCs to proliferate in response to PPD at a concentration of 10μg/ml was assessed using tritiated thymidine incorporation. No difference was found in the ability of young and old PBMCs to proliferate in response to antigen (Mann Whitney p=0.514).

3.2.3 Correlation of clinical score with peripheral blood responses

The proliferative responses of PBMCs were then correlated with clinical score in order to determine whether skin responses are reflective of peripheral blood responses (Figure 3.3). In young individuals, good PBMC proliferative responses (>10,000 cpm) were associated with a good clinical response to the MT. All young and old individuals who had a poor PBMC proliferative response (<10, 000cpm) to PPD at a dose of 10μg/ml had an absent clinical response. However, in the old group, there was dissociation between the observed absent clinical response in the skin and PBMC responses. In spite of good proliferative PBMC responses (equivalent to those seen in the young) old individuals failed to develop a clinical response to the MT (Figure 3.3).

This indicates that, in the old, the absent clinical response to the MT may not be reflective of global immunity to the antigen and may be reflective of age-specific changes in cutaneous immunity.

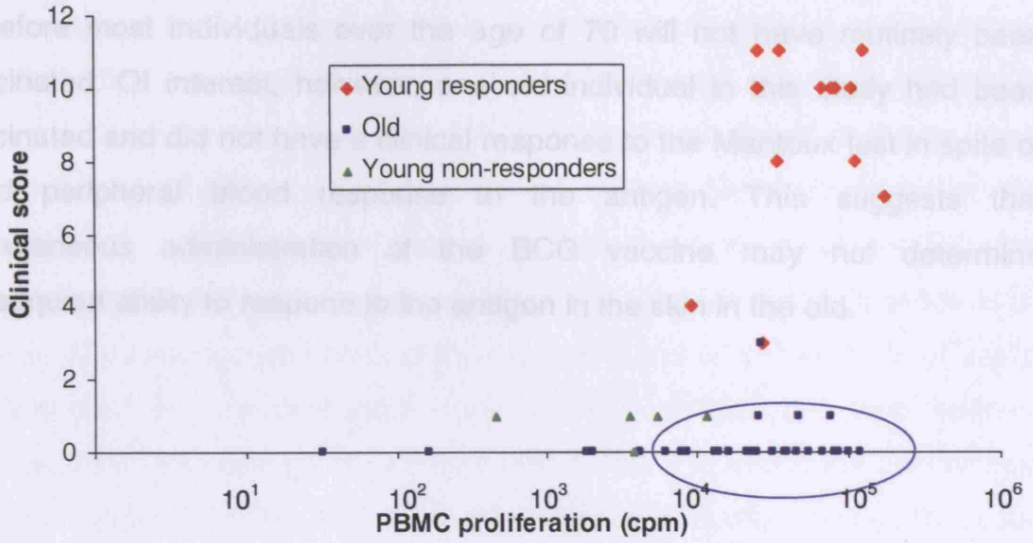


FIGURE 3.3. Correlation of the clinical score following the Mantoux test with PBMC proliferation.

The proliferative response of young and old PMBCs to PPD, as assessed by tritiated thymidine incorporation at Day 5, was correlated with the clinical score in the skin. In young individuals, good proliferative PBMC responses (>10000 cpm) were associated with a clinical response in the skin. However a large number of old individuals failed to develop a clinical response in the skin in spite of good PBMC responses (circled).

Another explanation for the observed differences between the young and old could be that all individuals in the young group had been immunised with the BCG (Bacillus of Calmette and Guerin) vaccine whereas only one individual in the old group had been vaccinated. Previous studies have demonstrated that the route of primary exposure to antigen may determine the ability of different tissues to subsequently develop memory responses to that antigen. PPD comprises of a complex mixture of peptides and carbohydrates derived from *M. tuberculosis* and immunity can be acquired in several ways; namely previous and/or latent infection with TB, BCG vaccination and exposure to environmental mycobacteria. The young individuals were most likely to have

developed immunity to the PPD antigen as a result of BCG vaccination (administered percutaneously) whereas immunity in the old group was likely to be due to previous exposure to tuberculosis or environmental mycobacteria. The BCG immunisation program in the UK did not start until the 1940s and therefore most individuals over the age of 70 will not have routinely been vaccinated. Of interest, however, one old individual in this study had been vaccinated and did not have a clinical response to the Mantoux test in spite of good peripheral blood response to the antigen. This suggests that percutaneous administration of the BCG vaccine may not determine subsequent ability to respond to the antigen in the skin in the old.

3.3 Candida skin testing

3.3.1 Clinical and peripheral blood responses to Candida antigen

In order to exclude differences in exposure to antigen between the young and old groups it was decided to skin test individuals with *Candida albicans* antigen as it was thought that the route and degree of exposure to this antigen was likely to be equivalent in both groups. Although *Candida albicans* is rarely recovered from normal skin, it is a common inhabitant of the mucosal surfaces of the oropharynx and gastrointestinal tract. Serological studies also indicate that a significant proportion of individuals negative for Candida colonisation at the time of testing have been exposed to Candida in the past. Superficial cutaneous infections of the skin are common in healthy individuals, in particular in occluded sites such as the axillae, toe web spaces, inframammary skin and groins where a warm, moist environment encourages colonisation and infection. Reduced cell mediated immunity, such as that seen in individuals with congenital T cell defects, further increases the risk of Candida infection as seen in chronic mucocutaneous candidiasis where extensive, multiple sites become infected[283;284].

3.3.2 Clinical response to Candida antigen

A total of 27 young and 23 old individuals were tested with the Candin skin test with intradermal injection of Candin skin test solution into the inner aspect of the upper forearm. A dose of 0.02ml of the Candin skin test solution was selected for use in the study as this was the smallest dose/volume that could be administered accurately intradermally without the need for diluting the commercial preparation with an associated risk of contamination of the skin test preparation. Once again, a clinical score was assigned for each individual tested based on erythema, diameter and palpability of the clinical response (Figure 2.2). In both young and old groups, a peak in clinical response to the Candin skin test was seen at 48- 72 hours. As with the MT, the clinical score was found to correlate with each individual parameter measured in both the young and old groups.

A marked reduction in clinical response to antigen injection was observed in the old (Mean: 1.65, Median:0) compared with the young (Mean: 5.52,

Median: 5) at Day 3 (Mann Whitney test P=0.0004) (Figure 3.4) and at all time points following the injection of 0.2ml of Candin skin test solution.

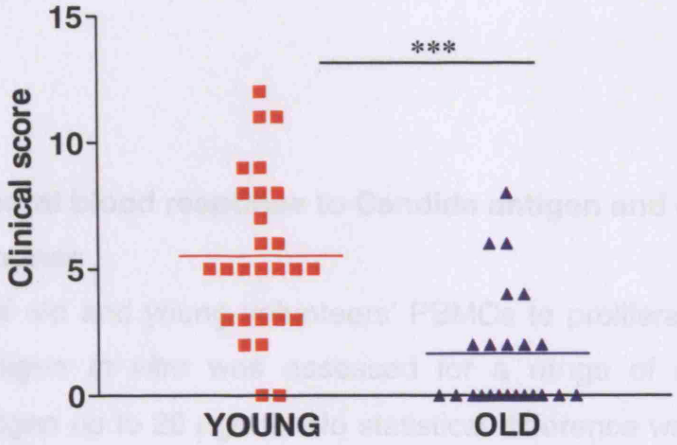


FIGURE 3.4. Clinical response to the injection of Candin skin test solution in the young and old.

There was a marked reduction in the clinical score at Day 3, following the injection of 0.02ml Candin skin test solution, in the old compared with the young (Mann Whitney p<0.0001).

Repeated testing of the skin with the PPD antigen has previously been reported to have a boosting effect on cutaneous responses[285]. One old volunteer was tested on 3 separate occasions with 0.02ml of Candin skin test solution, at separate sites on both forearms, several months apart. No clinical response was seen after the first skin test with 0.02ml Candin, in spite of good PBMC proliferative responses. However, on subsequent testing a positive clinical response was observed (clinical score=2) and this response increased further following the third skin test (clinical score=3). In view of these findings, intradermal injection of antigen was performed only once per individual for each skin test antigen.

It is also possible that there may be a threshold effect in the old, in that if sufficient quantities of antigen are injected into the skin a clinical response can be induced. The use of a higher dose of antigen, however, may not be truly

reflective of antigen load *in vivo.* In 3 old individuals with good PBMC responses to candida, a higher skin test dose of 0.1ml of Candin was used for skin testing. Of the old individuals tested, 2 of 3 developed a clinical response (Mean clinical score=4.3).

3.3.3 Peripheral blood response to Candida antigen and correlation with clinical response

The ability of old and young volunteers' PBMCs to proliferate in response to Candida antigen *in vitro* was assessed for a range of concentrations of Candida antigen up to 20 μg/ml. No statistical difference was found between young and old PBMC proliferation at all concentrations of candida antigen tested including the 20 μg/ml concentration (Mann Whitney p= 0.69) (Figure 3.5A). The clinical response to the skin test was then correlated with PBMC proliferative responses. This demonstrated that, as with the MT, there were significant numbers of old volunteers who failed to respond clinically to Candida antigen injection in spite of good peripheral blood responses (Figure 3.5B). The majority of young individuals tested had both good peripheral blood responses and skin responses to the Candida antigen. Only one individual in the young group failed to develop a clinical response in spite of good peripheral blood responses. No specific reason for this could be identified.

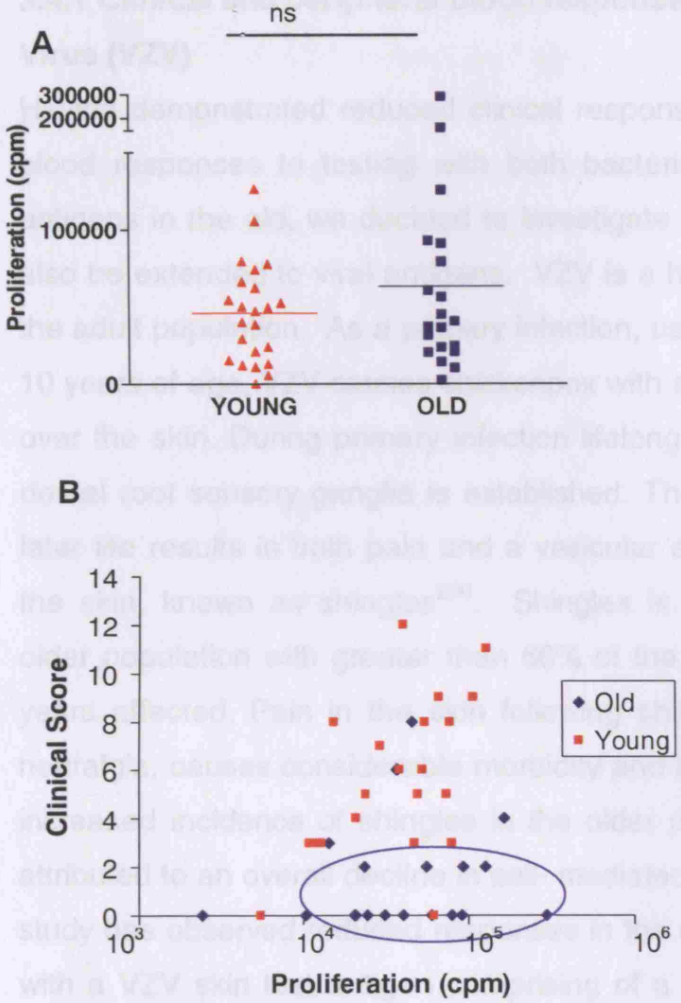


FIGURE 3.5. Proliferation of PBMCs in response to Candida antigen and correlation with the clinical response to the Candin skin test in the young and old.

A. Proliferative PBMC responses were assessed by tritiated thymidine incorporation after 6 days in culture with the Candida antigen at a concentration of 20μg/ml. No difference in PBMC proliferation was found between the young and old groups (Mann Whitney p=0.69).)Linear scale shown for proliferation.

B. As for PPD, the clinical response in the skin at Day 3 to the injection of Candin skin test solution was correlated with PBMC proliferative responses to Candida antigen (20μg/ml). In the young a good PBMC response was associated with a good clinical response in the skin. In the old however there was a group of individuals who failed to develop a clinical response in the skin in spite of good PBMC responses (circled).

3.4 Varicella zoster virus (VZV) skin testing

3.4.1 Clinical and peripheral blood responses to the Varicella Zoster Virus (VZV)

Having demonstrated reduced clinical responses in spite of good peripheral blood responses to testing with both bacterial (PPD) and fungal (Candin) antigens in the old, we decided to investigate whether this observation could also be extended to viral antigens. VZV is a herpes virus that infects 98% of the adult population. As a primary infection, usually seen in children less than 10 years of age, VZV causes chickenpox with a widespread vesicular eruption over the skin. During primary infection lifelong infection within the cutaneous dorsal root sensory ganglia is established. The reactivation of latent virus in later life results in both pain and a vesicular eruption within a dermatome in the skin, known as shingles[286]. Shingles is particularly problematic in the older population with greater than 66% of the population over the age of 50 years affected. Pain in the skin following shingles, known as post herpetic neuralgia, causes considerable morbidity and often persists long term[141]. The increased incidence of shingles in the older population has previously been attributed to an overall decline in cell- mediated immunity with age. A previous study has observed reduced responses in the skin of old individuals to testing with a VZV skin test antigen comprising of a heat inactivated OKA strain of VZV[287]. However clinical responses in the skin were not correlated with underlying responses to VZV antigen in the peripheral blood.

0.02ml of VZV skin test solution was injected intradermally into the inner aspect of the forearm in 8 young and 10 old individuals. As for the Candida skin test, the 0.02ml volume of skin test solution was selected on the basis that this was the smallest volume that could accurately be injected into the skin. A reduction in the clinical score was observed in the old group (Mean=1.7, Median:0) compared with the young (Mean= 6.38, Median:6.00) (Mann Whitney Test p=0.0021) (Figure 3.6A). PBMC responses to VZV antigen were also assessed using proliferation assays. Purified PBMCs were cultured with VZV antigen over a range of concentrations up to 4μl/ml for 6 days and proliferation was then assessed using tritiated thymidine

incorporation. Unlike proliferative responses to Candida and PPD antigens, there was a reduction in PBMC proliferation in the old for a range of concentrations of VZV antigen, including proliferation at a peak concentration of 4μl/ml Figure 3.6B)(Mann Whitney test p=0.04) This suggests that older individuals lose systemic immunity to the VZV virus and this may explain the increased frequency of viral reactivation in the old.

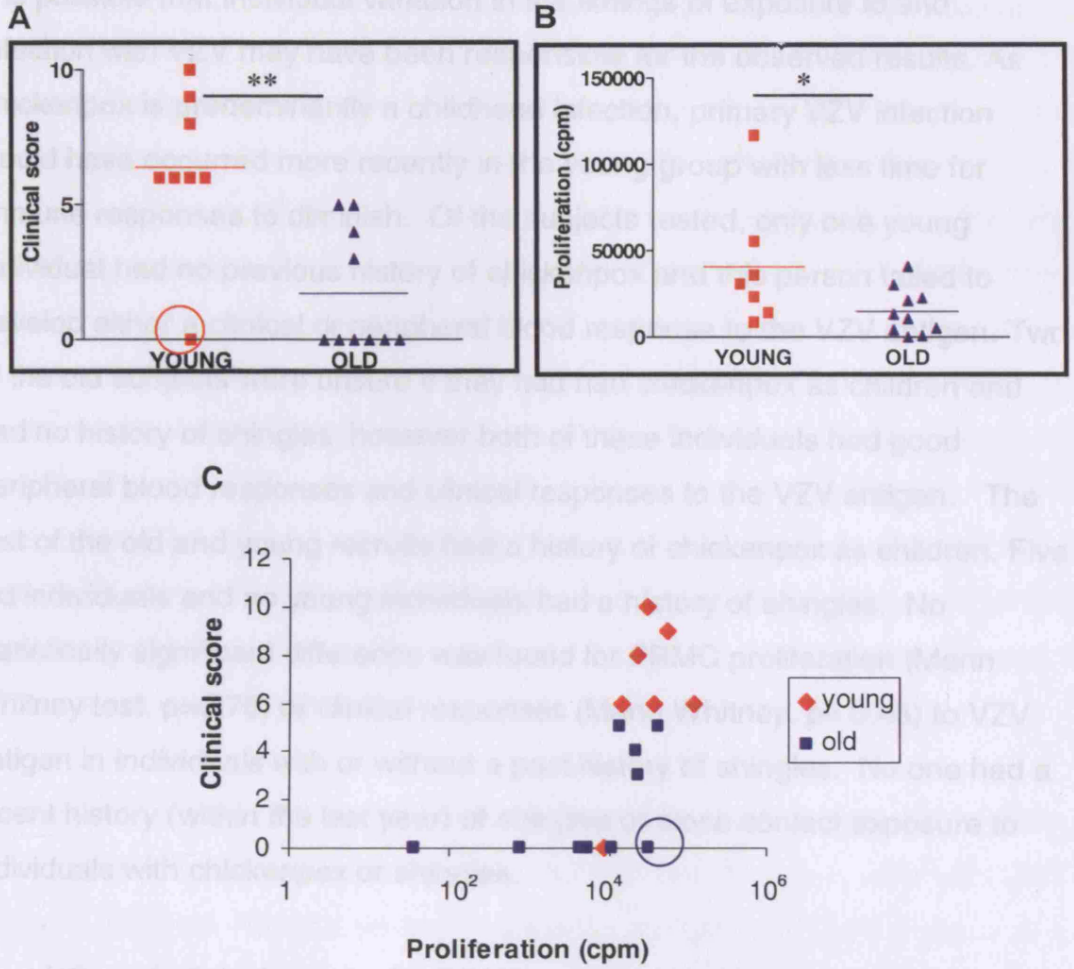


FIGURE 3.6 Proliferation of PBMCs in response to VZV antigen and correlation with the clinical response to the VZV skin test in the young and old.

A. A significant reduction in the clinical score in the skin was seen in the old compared with the young at Day 3 after injection of 0.02ml of the VZV skin test solution (p=0.0021). Only one young individual, who had no previous history of chickenpox, failed to respond to the VZV skin test (circled).

B. Following 6 days of *in vitro* culture of PBMCs with the VZV antigen, PBMC proliferation was assessed using tritiated thymidine incorporation. A reduction in proliferative responses at a peak concentration of 4μl/ml was observed in the old compared with the young (Mann Whitney test p=0.04).

C. PBMC proliferative responses to VZV antigen were correlated with the Day 3 clinical score following injection of VZV skin test antigen. One old individual was found to have an absent skin response in spite of good PBMC proliferative responses to VZV antigen (circled).

It is possible that individual variation in the timings of exposure to and infection with VZV may have been responsible for the observed results. As chickenpox is predominantly a childhood infection, primary VZV infection would have occurred more recently in the young group with less time for immune responses to diminish. Of the subjects tested, only one young individual had no previous history of chickenpox and this person failed to develop either a clinical or peripheral blood response to the VZV antigen. Two of the old subjects were unsure if they had had chickenpox as children and had no history of shingles, however both of these individuals had good peripheral blood responses and clinical responses to the VZV antigen. The rest of the old and young recruits had a history of chickenpox as children. Five old individuals and no young individuals had a history of shingles. No statistically significant difference was found for PBMC proliferation (Mann Whitney test. p=0.76) or clinical responses (Mann Whitney. p= 0.48) to VZV antigen in individuals with or without a past history of shingles. No one had a recent history (within the last year) of shingles or close contact exposure to individuals with chickenpox or shingles.

Correlation of clinical score with PBMC proliferation revealed that, as for the other skin test antigens, all young individuals with a good blood response to VZV antigen developed a good clinical response to the VZV skin test (Figure 3.6C). Four old individuals had a detectable clinical response to injection with the VZV antigen in addition to good peripheral blood responses however these scores were significantly reduced compared with the young responder group (Mann Whitney test. p=0.006). One old individual failed to develop a clinical skin response in spite of a good PBMC proliferative response to VZV antigen (Figure 3.6C). Due to the limited numbers of subjects tested it is difficult to conclude what proportion of old individuals are unresponsive to skin testing with VZV skin test antigen in spite of good peripheral blood responses, however a skin-specific loss of immunity in the old to VZV may in part explain the prediliction for cutaneous involvement following viral reactivation in shingles.

3.5 Effects of cytomegalovirus (CMV) infection on immune responses

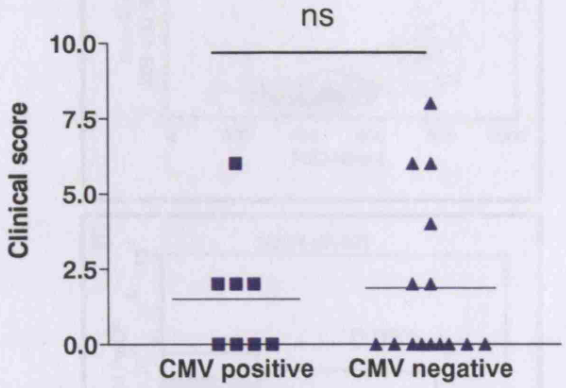
Loss of immunity to certain antigens, for example VZV, with ageing may be related to infection with other chronic latent viral infections such as human cytamegalovirus (CMV). CMV infection is thought to be one of the indicators of the so-called immune risk phenotype seen with ageing[132]. The massive clonal expansion of CMV specific T cells seen in individuals with chronic latent infection is thought to crowd out T cells with other antigenic specificities with a resulting restriction in the memory T cell pool repertoire[174]. It has previously been demonstrated that there is a reduction in telomere length of antigen-specific T lymphocytes for a variety of antigens including VZV and PPD in individuals with latent CMV infection[173]. Once telomeres have reached a critical length the cell will undergo apoptosis. A reduction in telomere length therefore implies that there is a reduced replicative capacity and therefore reduced immunity to the antigen for which the T cells are specific.

CMV positive individuals in this study were identified on the basis of the ability of their PBMCs to produce IFN-γ after overnight incubation *in vitro* with CMV antigen. Purified PBMCs were stimulated overnight with CMV antigen at a final concentration of 100 μl/ml in the presence of Brefeldin A. IFN-γ producing cells were then identified using fluorescently labelled antibodies and analysis by flow cytometry. (Figure 3.8). We have previously established that individuals with a positive IFN-γ overnight incubation response to antigen also have serum IgG antibodies to CMV, indicating that these individuals have persistent latent viral infection.

The clinical DTH response to Candin skin test solution (Figure 3.7A) and PPD in the skin did not appear to be affected by a positive CMV status in the young or old groups. Of the 4 old individuals that had a clinical response to testing with PPD, 50% were CMV positive. However, a higher proportion of CMV positive compared with CMV negative individuals failed to develop a clinical response to VZV in the old group (83% of CMV positive individuals versus 25% of CMV negative individuals)(Figure 3.7B). This result may also reflect the fact that overall PBMC responses were reduced in the CMV positive group

compared with the CMV negative group (mean cpm 11154 vs 20481 respectively). The number of individuals tested was too small for the results to reach statistical significance. CMV status did not influence response to VZV testing in the young, however it is thought that chronicity of CMV infection is important for exerting detrimental effects on the immune system and so the young individuals' immunity may as yet be unaffected by CMV infection.

A. CANDIN SKIN TEST



B. VZV SKIN TEST

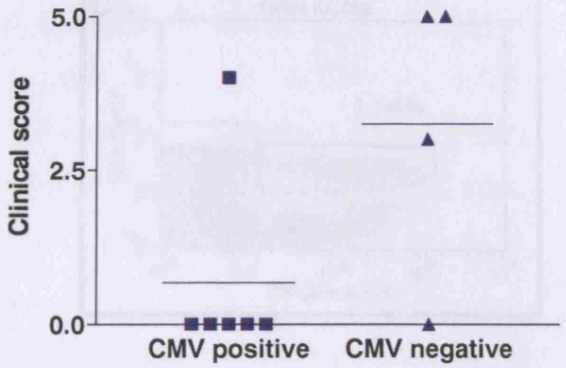


FIGURE 3.7 Effect of CMV infection on the clinical response to Candin and VZV skin test responses in the old.

A. No difference in clinical score at Day 3 following Candin skin test injection was found between old individuals with and without CMV infection. Infection with latent CMV in the individuals studied was determined by ability of CD4+ T lymphocytes to produce IFN-γ in response to overnight stimulation with CMV antigen in the presence of brefeldin A, as determined by flow cytometry (Figure 3.8). **B.** The clinical score at Day 3 following VZV skin test injection was compared in old individuals with and without CMV infection. No statistically significant difference was identified between CMV positive and negative individuals (Mann Whitney p=0.11), however a larger proportion of old individuals with CMV had an absent response to VZV skin test (83%), compared with the CMV negative (25%) individuals.

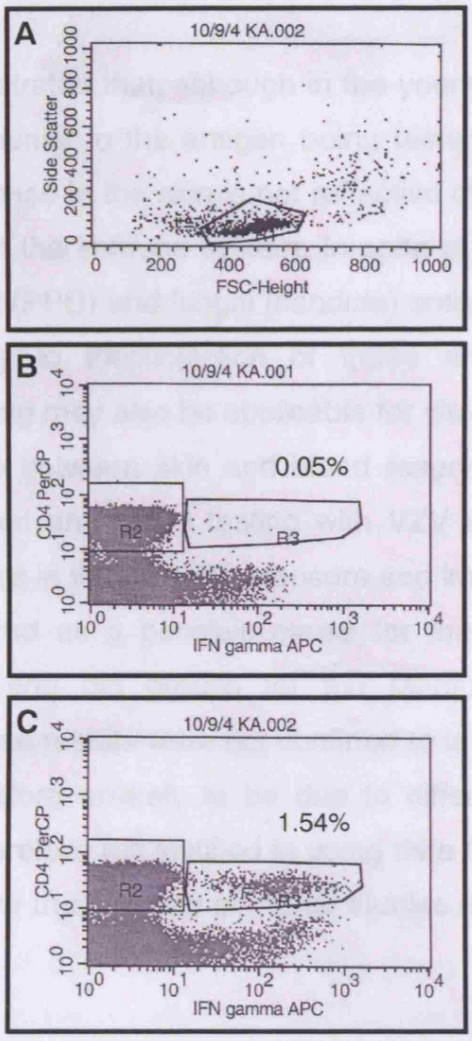


FIGURE 3.8 PBMC- derived CD4+ T lymphocyte intracellular IFN-γ expression following culture with CMV antigen.

PBMCs were incubated for 15 hours with or without CMV antigen in the presence of Brefeldin A that was added after the first 2 hours of culture. The cells were then stained with CD4 PercP and IFN-γ APC following fixation and permeabilisation (Caltag Fix and Perm Cell kit). The cells were examined for IFN-γ expression by gating on live lymphocytes based on FSC/SSC profile **(A)** and then CD4 expression (R2 and R3 gates). The dot plots show representative expression of IFN-γ in CD4+ cells in the unstimulated control sample **(B)** and following addition of the CMV antigen **(C)** (The percentage of IFN-γ+ cells in this representative individual is shown). Percentages of antigen specific cells were calculated by subtracting the percentage of IFN-γ positive cells present in the unstimulated cell sample (B) from the CMV stimulated sample(C).

3.6 Discussion

This work has demonstrated that, although in the young DTH skin testing is a good indicator of immunity to the antigen being tested, in the old group the diminished DTH response in the skin is not reflective of a comparable decline in all compartments of the immune system. In spite of good peripheral blood responses to bacterial (PPD) and fungal (candida) antigens, old individuals do not respond clinically to the injection of these antigens into the skin. Furthermore, this finding may also be applicable for viral recall antigens in that the same dissociation between skin and blood responses was seen in one individual following skin and blood testing with VZV antigen. Although the possibility of differences in the route of exposure and immunisation to the PPD antigen was considered as a possible cause for the observed differences between the young and old groups for the Mantoux test, our findings demonstrated that these results were not confined to testing with PPD antigen alone and were therefore unlikely to be due to differences in the route of immunisation. We therefore felt justified in using data from both Candida and Mantoux skin testing for the purpose of further studies discussed in this thesis.

It is well recognised that certain groups of young individuals who are immunosuppressed, such as those with HIV, may have a negative MT in spite of acute infection with TB[288]. Numerous studies have documented age-associated changes in different types of immune cells including T cells, B cells and antigen presenting cells that may result in defective immunity with ageing[191]. In particular, changes in the memory T cell pool may result in the loss of immune memory[173]. However, the equal ability of young and old PBMCs to proliferate in response to both PPD and candida antigens suggests that healthy old individuals used in this study were not significantly immunocompromised. It is possible that PBMC proliferation is not totally representative of an individuals' ability to respond to infection. However, previous studies investigating global parameters of immunity in healthy old individuals also indicate that the decline in immunity is modest in this population[289]. This data is further supported by the prior observation that anergic old individuals have been able to mount an adequate secondary

immune response following infection with acute TB[290]. A negative skin test response to cutaneous challenge with antigen in the old is therefore not necessarily reflective of globally reduced immunity.

The use of the Mantoux test to assess for BCG vaccine efficacy as a correlate of protective immunity is questionable. A previous study has indicated that some individuals that have been vaccinated with BCG whilst young lose their ability to respond to skin testing with time[56]. With the increase in worldwide tuberculosis infection and the advent of immunomodulatory anti- TNF α drugs to treat disease such as rheumatoid arthritis and psoriasis, where there is a risk of reactivation of latent TB[291-293], it is essential that we have the tests available to be able to accurately assess old individuals' immunity to TB. These findings show that the Mantoux test greatly underestimates immunity in healthy old individuals and therefore this study has wide reaching implications for the use of the Mantoux test as a screening test.

The diameter of the response (>15mm) is often used to determine the likelihood of a positive response due to BCG vaccination versus acute or latent TB infection. Our findings suggest that this method may not be reliable in old individuals. More recently, the use of Elispot assays assessing IFN-γ peripheral blood responses to 2 antigens specific for the human tuberculosis; early secretory antigenic target 6 (ESAT 6) and culture filtrate protein 10 (CFP10)) have been developed[294] and may represent a more accurate and reliable method for screening individuals of all ages for infection with TB.

One of the major limitations to longevity with a good quality of life is likely to be related to the ability of the immune system to function adequately[197] and therefore tests providing accurate information on immunity to infection are going to become increasingly important as the proportion of individuals over the age of 70 within the population increases. In view of limited financial resources, the identification of individuals that would benefit from boosting of their immunity to specific infections, for example by immunisation to influenza

or VZV, is desirable. However, this work indicates that skin testing is not suitable for this purpose in the old.

The apparent dissociation between cutaneous and blood responses to antigen suggests that there is a skin specific reduction in immunity that occurs with ageing. These findings have wide reaching implications in that they may explain the observed increase in cutaneous malignancy[150], infection and reduction in severity of atopic dermatitis with age[295]. The potential mechanisms for skin-specific reduction in immunity are the focus of further investigation discussed in this thesis.

4. Characterising the lymphocytic response to antigen in the old.

4.1 Introduction

In humans, the skin represents an accessible organ in which we can readily study dynamic immune responses. Immunohistochemistry and flow cytometry techniques were used in this study in order to characterise the *in vivo* dynamics of lymphocytic responses in the skin during the course of a DTH response to both the MT and Candin. Both techniques are complimentary, in that whilst immunohistochemistry permitted the morphological and quantitative investigation of cell types present in the skin, flow cytometry allowed for more accurate multiparameter phenotyping of cells in addition to assessment of functional characteristics.

Various cell isolation techniques, such as enzyme digestion and mechanical disaggregation, have been employed by different investigators in the past in order to retrieve skin cells suitable for flow cytometric analysis. However, reduced expression of CD3, CD4 and CD8 in addition to integrins and selectins on lymphocytes has been reported with the use of such techniques. Skin explant cultures can also be used to extract lymphocytes from the skin, however this requires 5 days or longer of *in vitro* culture with possible resulting changes in cell phenotype, limiting the study of dynamic responses in the skin. In this study, a skin suction blister technique was used to extract viable T lymphocytes accumulating at the site of the DTH response for the purpose of flow cytometry. This technique allowed for direct *ex-vivo* study of cell phenotype and function in addition to the collection of fluid in order to study chemical mediators present at the site (discussed in Chapter 5).

The lymphocytic response during the course of the Mantoux reaction in the young has been characterised. This has revealed that the lymphocytic response comprises predominantly of CD4+ memory (CD45RA-) T lymphocytes that proliferate and accumulate at the site of the response, reaching a peak at Day 7[64]. In addition, there is an expansion and contraction

of an antigen specific cell population of CD4+ T lymphocytes during the course of the response[88]. Interestingly, there is dissociation between the peak clinical response (seen at 48-72 hours) and the peak lymphocytic response (seen at Day 7)[64]. Although other cell types, such as macrophages and neutrophils are seen in abundance in the skin at early time points, a clinical response is only seen in those with immunity to the antigen indicating that adaptive, cell mediated immunity is essential for a clinical response to develop in the skin. This suggests that T lymphocytes must play a crucial and early role in the development of the clinical response following the intradermal injection of recall antigens.

We therefore investigated whether the observed reduction in clinical response to antigen injection in the old was also associated with a reduced lymphocytic response in the skin.

4.2 Number of T lymphocytes at site of response in skin

4.2.1 Cell numbers retrieved from skin suction blisters following the MT using the Trucount technique.

Skin suction blisters were raised over the site of the MT in 3 old and 3 young volunteers at Day 7. The Day 7 time point was chosen as this has previously been shown to be the time of peak lymphocytic infiltration into the skin following the MT in the young[64]. The old volunteers selected for the study had no clinical response to the MT in spite of good peripheral blood responses to PPD. The young group had good clinical responses to the MT in addition to good peripheral blood responses to PPD.

Cells were separated from the blister fluid by microcentrifugation and total numbers of CD3+ and CD4+ lymphocytes were calculated using the Trucount technique. A reduction in the number of CD3+ and CD4+ T lymphocytes retrieved from the blister fluid was seen at Day 7 in the old subjects compared with the young (Figure 4.1) (Mann Whitney p=0.02) for both CD3 and CD4 T lymphocytes.

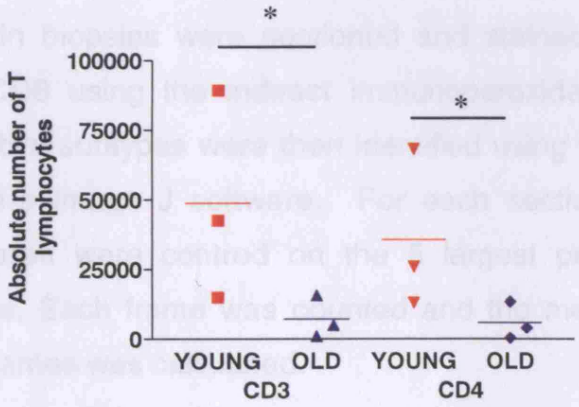


FIGURE 4.1 Numbers of CD3+ and CD4+ lymphocytes in young and old blisters at the site of the MT.
Skin suction blisters were raised over the site of the MT at Day 7 in young and old. Cells isolated from blister fluid were stained with cell surface antibodies to CD3 and CD4 and counted using the Trucount technique. A significant reduction in both numbers of CD3 and CD4 T lymphocytes were found in the old compared with the young (Mann Whitney p=0.02 for both CD3+ and CD4+ T lymphocytes).

4.2.2 Cell numbers counted from skin biopsies following Candin skin test (CD3+, CD4+, CD8+ cells)

In order to assess whether a similar reduction in cell number was observed with other antigens, we decided to investigate the cellular response to Candin skin tests. Although we had demonstrated a reduction in numbers of lymphocytes isolated from skin suction blisters in the old for the MT, we were unable to exclude the possibility that not all of the cells present in the skin were retrieved using the skin suction blister technique. In view of this, it was decided to analyse cell numbers on the basis of analysis of skin biopsies. 5mm skin punch biopsies were taken from the site of injection of 0.02ml Candin skin test solution at day 0, 1, 3 and 7 in young and old individuals. The clinical score was recorded at day 3 and at the time of biopsy. A minimum of 4 young and 4 old individuals were tested at each time point. In the young and old groups there was no significant difference in the mean clinical response scores obtained at 72 hours for the groups at each of the time points, confirming that similar clinical responses were obtained regardless of the time of the biopsy (Kruskal-Wallis young: $p=0.48$, old: $p=0.32$)

Fresh frozen skin biopsies were sectioned and stained with antibodies for CD3, CD4 or CD8 using the indirect immunoperoxidase technique. The numbers of positive subtypes were then identified using light microscopy and counted using the Image J software. For each section, 5 frames with a predetermined area were centred on the 5 largest perivascular infiltrates within the dermis. Each frame was counted and the mean value of the cell count for the 5 frames was calculated.

Normal skin was found to contain small numbers of lymphocytes, predominantly seen around blood vessels, in both the young and old groups (Figure 4.2). No significant difference was found between numbers of cells in the young and old (Mean young vs old: CD3:1.56 cells(SD±1.88) vs 1.64 cells(SD±1.87), CD4:0.56 cells(SD±0.71) vs 1.56 cells(SD±1.39), CD8: 0.13 cells(SD±0.14) vs 0.25 cells (SD±0.23). In both groups CD4+ cells outnumbered CD8+ cells (ratio of 4.6:1 CD4:CD8 cells combined data from

old and young groups). It is possible that a proportion of the CD4+ cells stained represented resident tissue macrophages, however the sum total of CD4+ and CD8+ T cells equalled the number of CD3+ T lymphocytes in most cases, suggesting the number of non- lymphocytic cells expressing CD4 in normal skin were negligible.

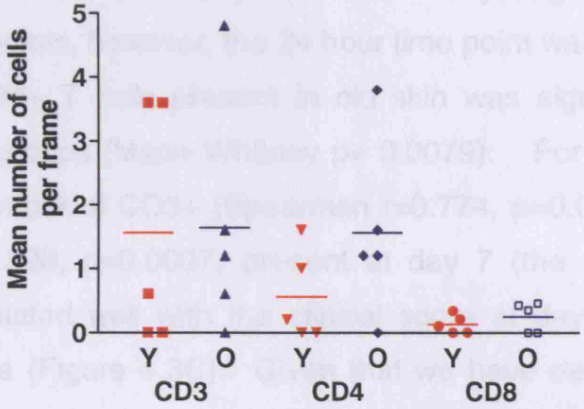


FIGURE 4.2 Numbers of CD3+, CD4+ and CD8+ in normal skin in young and old.

Skin biopsies taken from normal skin on the forearm were stained with antibodies for CD3, CD4 or CD8 using the immunoperoxidase technique in young (**Y**) (Photo plate 1) and old (**O**) individuals. Cell numbers are expressed as the mean number of cells counted for 5 representative frames for each individual. No significant difference in the numbers of cells was found for all cell types (Mann Whitney CD3: p=0.69, CD4: p=0.22, CD8: p=0.42) between the young and old groups. In both young and old groups a higher numbers of CD4+ cells were seen compared with CD8+ cells.

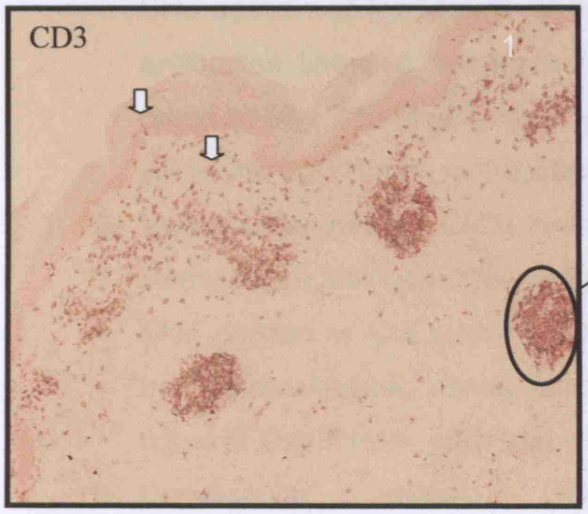
4.2.3 Numbers of CD3+ and CD4+ cells

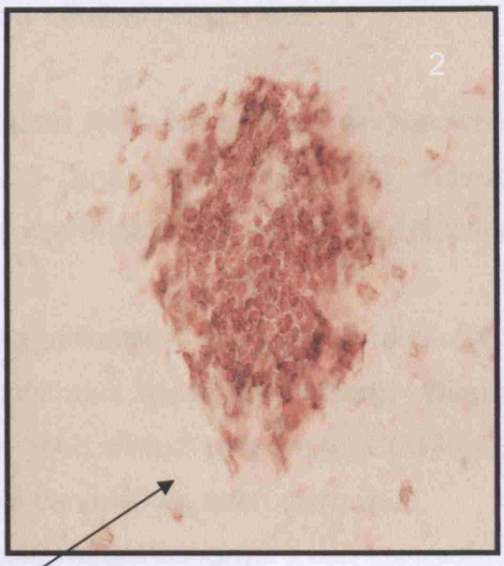
In the young, after the injection of intradermal Candin, CD3+ and CD4+ cells were seen to accumulate perivascularly within the dermis (Photo plate 1). Perivascular CD4+ and CD3+ cell numbers had risen significantly from baseline by 24 hours (p=0.0079 for both CD3+ and CD4+ cells) with subsequent increases in the number of cells at day 3 (mean=CD4+:52.6 cells SD±21.93, CD3+:77.0 cells SD±39.68) and day 7(mean=CD4+ 70.68 cells

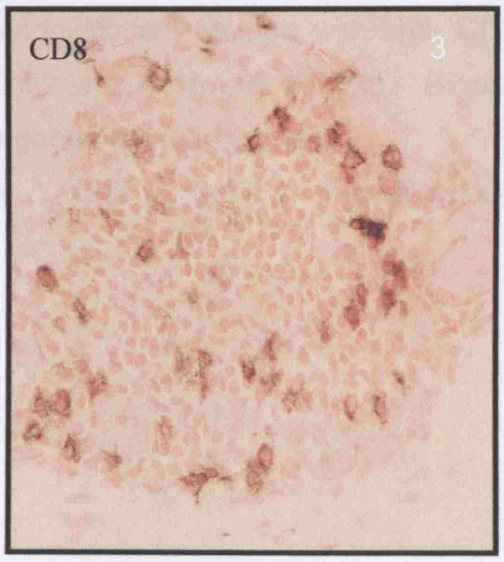
SD±36.69, CD3+ 92.25 cells SD±46.43). Although the majority of infiltrating cells were within perivascular areas, small numbers of cells were seen within the interstitium and the epidermis (Photo plate 1) in some individuals. This was particularly evident where the total number of lymphocytes infiltrating the skin was large.

A reduced mean number of CD4+ and CD3+ T cells was seen over all time points studied in the old compared with the young (Fig 4.3A+B). For individual time points, however, the 24 hour time point was the only time that a reduction in CD4+ T cells present in old skin was significant between the young and old groups (Mann Whitney p= 0.0079). For both young and old biopsies the number of CD3+ (Spearman r=0.774, p=0.017) and CD4+ cells (Spearman r=0.928, p=0.0007) present at day 7 (the peak of the cellular response) correlated well with the clinical score at day 3 (the peak of the clinical response (Figure 4.3C). Given that we have demonstrated reduced clinical score in the old group as a whole it is therefore likely that cell numbers present in the skin are reduced overall in the old group.

CD3, CD4 and CD8 STAINING







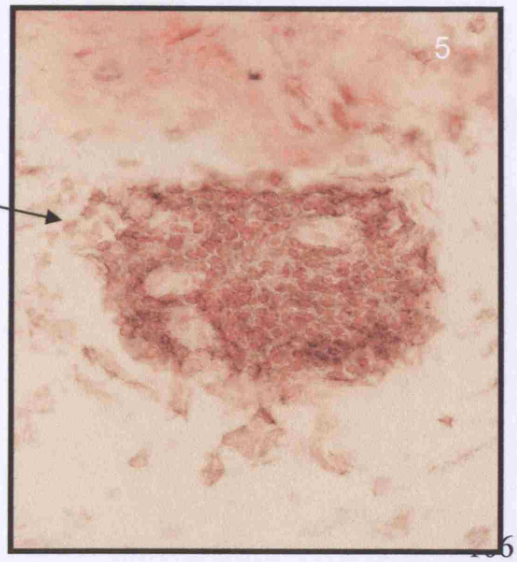
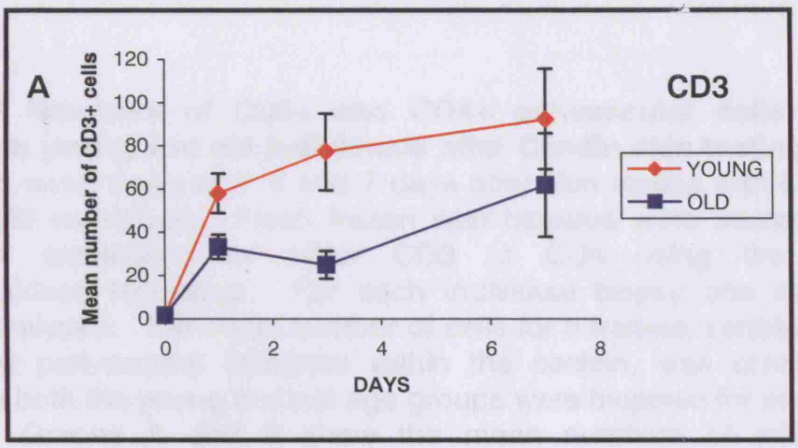
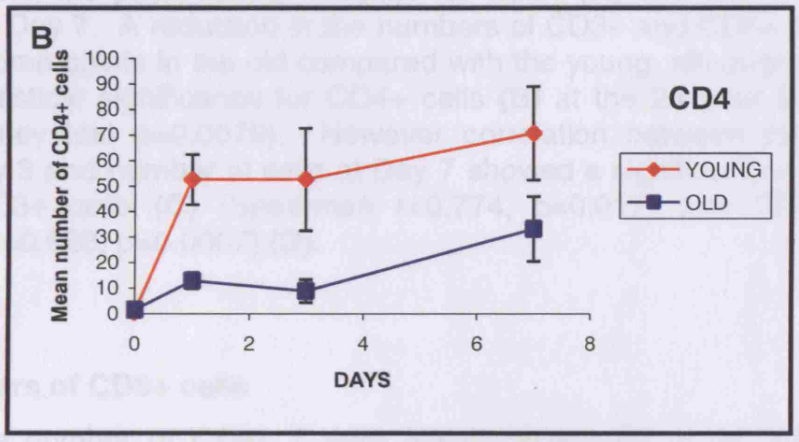


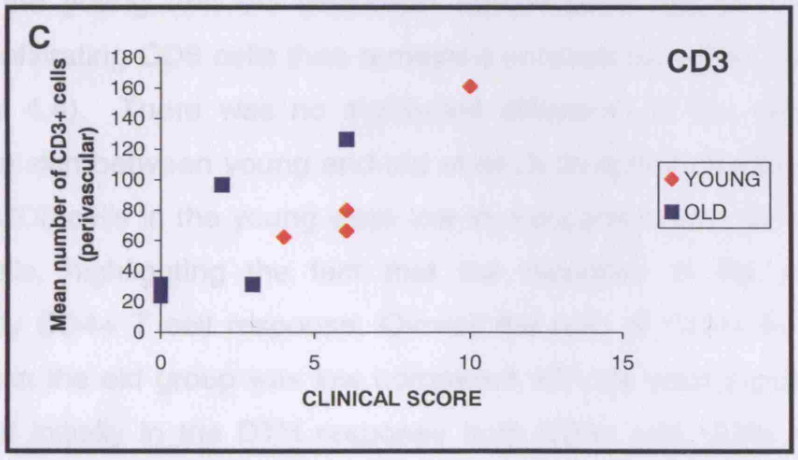
PHOTO PLATE 1. Infiltration of CD4, CD3 and CD8+ cells at the site of antigen injection in the young with good clinical responses.

Fresh frozen sections of skin biopsies taken from the site of injection were stained with antibodies for CD4, CD3 and CD8 using the indirect immunoperoxidase technique. Sections were counterstained with Nuclear Fast Red.

1. Skin section at low power (x10 magnification) stained with anti- CD3 antibodies showing perivascular infiltrates (example circled). Biopsy taken at Day 7 from individual with good clinical response. CD3+ cells within the interstitium and epidermis can also be seen (arrowed).
2. Perivascular infiltrate CD3+ cells (x40 magnification).
3. Perivascular infiltrate CD8+ cells (x40 magnification).
4. Skin section at low power (x10 magnification) stained with anti- CD4 antibodies showing perivascular infiltrates (example circled). Biopsy taken at Day 7 from individual with good clinical response.
5. Perivascular infiltrate CD4+ cells (x40 magnification).


A
CD3
Mean number of CD3+ cells
120
100
80
60
40
20
0
0
2
4
6
8
DAYS
YOUNG
OLD


B
CD4
Mean number of CD4+ cells
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
DAYS
YOUNG
OLD


C
CD3
Mean number of CD3+ cells (perivascular)
180
160
140
120
100
80
60
40
20
0
0
5
10
15
CLINICAL SCORE
YOUNG
OLD

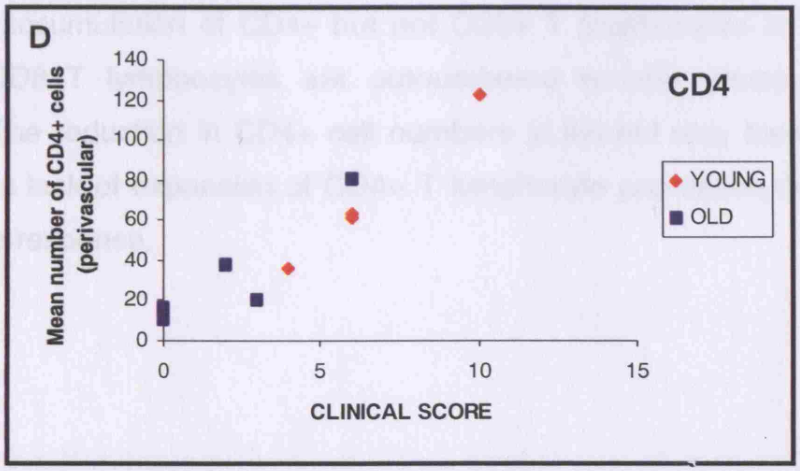

D
CD4
Mean number of CD4+ cells (perivascular)
140
120
100
80
60
40
20
0
0
5
10
15
CLINICAL SCORE
YOUNG
OLD

FIGURE 4.3

FIGURE 4.3 Numbers of CD3+ and CD4+ perivascular cells in skin biopsies from young and old individuals after Candin skin testing.
Skin biopsies were taken at 1, 3 and 7 days after skin testing with Candin in young and old individuals. Fresh frozen skin biopsies were sectioned and stained with antibodies for either CD3 or CD4 using the indirect immunoperoxidase technique. For each individual biopsy one section of tissue was analysed. The mean number of cells for 5 frames, centred around the 5 largest perivascular infiltrates within the section, was calculated. 5 individuals in both the young and old age groups were biopsied for each of the time points. Graphs A and B show the mean numbers of cells within perivascular infiltrates for each individual. Error bars show the SEM. In the young and old, the peak cellular infiltrate for CD3+ **(A)** and CD4+ cells **(B)** was seen at Day 7. A reduction in the numbers of CD3+ and CD4+ cells was seen for all time points in the old compared with the young, although this only reached statistical significance for CD4+ cells **(B)** at the 24 hour time point (Mann Whitney test p=0.0079). However correlation between the clinical score at Day 3 and number of cells at Day 7 showed a significant correlation, for both CD3+ cells **(C)** (Spearman r=0.774, p=0.017) and CD4+ cells (Spearman r=0.928, p=0.0007) **(D).**

4.2.4 Numbers of CD8+ cells

Although the number of CD8+ T cells initially increased at 24 hours from baseline in the young and old (Wilcoxon ranked pairs test p=0.002), the numbers of infiltrating CD8 cells then remained constant up to the Day 7 time point (Figure 4.4). There was no significant difference in the numbers of CD8+ cells in skin between young and old at each timepoint measured. The numbers of CD8 cells in the young were low in comparison with the numbers of CD4+ cells, highlighting the fact that the response in the skin is a predominantly CD4+ T cell response. Overall the ratio of CD4+ to CD8+ T lymphocytes in the old group was low compared with the young group. This suggests that initially in the DTH response both CD4+ and CD8+ cells are recruited to the skin in the young and old. In the young, however, there is subsequent accumulation of CD4+ but not CD8+ T lymphocytes at the site, such that CD8+T lymphocytes are outnumbered in later phases of the response. The reduction in CD4+ cell numbers in the old may therefore be reflective of a lack of expansion of CD4+ T lymphocyte population during the course of the response.

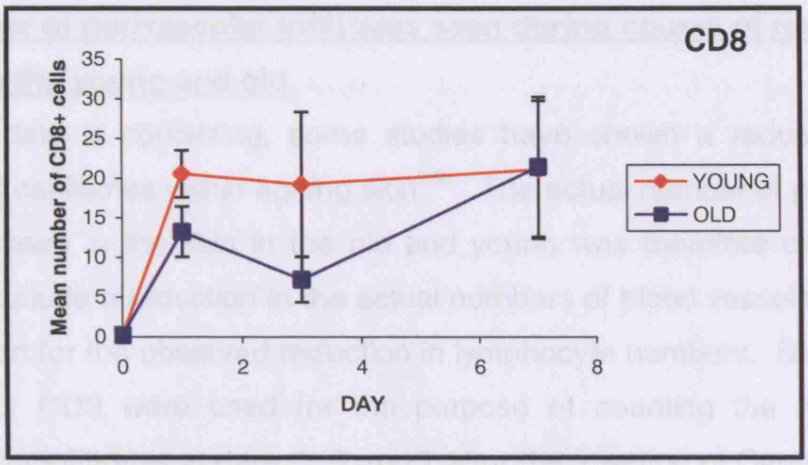


FIGURE 4.4 Numbers of CD8+ perivascular cells in skin biopsies taken from young and old individuals after Candin skin testing.

Skin biopsies were taken at 0, 1, 3 and 7 days after skin testing with Candin in the old and young. 4-5 different individuals were tested for both groups at each time point. Biopsies were sectioned and stained with an antibody for CD8 using the indirect immunoperoxidase technique. For each individual, 5 frames centred around the 5 largest perivascular infiltrates were counted per section and the mean number of cells was calculated. Although at 24 hours there was a significant increase in the number of CD8+ cells in the skin in both the young and old groups (Wilcoxon ranked pairs test $p=0.002$ for combined data from both groups), the number of CD8+ cells then remained relatively constant up to the Day 7 time point. No difference in the number of CD8+ cells was found for each time point between young and old groups.

4.3 Number of perivascular infiltrates seen during course of response to Candin in the young and old.

Although data is conflicting, some studies have shown a reduction in the number of capillaries within ageing skin[258]. The actual number of perivascular infiltrates seen in the skin in the old and young was therefore calculated in order to exclude a reduction in the actual numbers of blood vessels in old skin as a reason for the observed reduction in lymphocyte numbers. Skin sections stained for CD3 were used for the purpose of counting the numbers of perivascular infiltrates at days 1, 3 and 7 after the injection of Candin skin test solution into the skin. The number of infiltrates within 3 representative low power fields was counted for each skin section and the mean of the 3 counts was calculated (Figure 4.5). No significant change in the number of perivascular infiltrates present in the skin biopsies over the time course studied was found in either the old (Kruskall Wallis test $p=0.8$) or the young groups (Kruskall Wallis test $p=0.9$). In addition, no significant difference in the number of perivascular infiltrates was found between young and old skin, suggesting that there was no significant reduction in the actual number of blood vessels within the skin in the old.

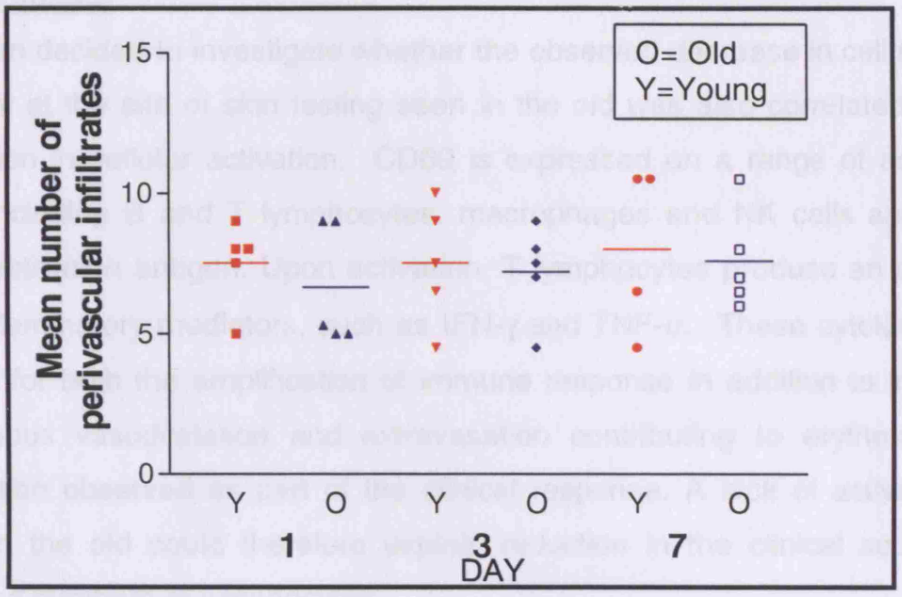


FIGURE 4.5 Numbers of perivascular infiltrates in skin biopsies from young and old individuals

Skin biopsies were taken at 1, 3 and 7 days after skin testing with Candin in the old and young. Biopsies were sectioned and stained with an antibody for CD3 using the indirect immunoperoxidase technique. Sections were also counterstained with Nuclear Fast Red. For each individual, 3 representative low power frames were selected and the number of perivascular infiltrates within each field was counted. The mean number of perivascular infiltrates per field was then calculated for each individual. In both young (Y) and old (O) individuals no change in the actual number of perivascular infiltrates seen occurred throughout the response (Kruskall Wallis test $p=0.8$ old, $p=0.9$ young). No significant difference in the number of perivascular infiltrates was found between old and young individuals. (Mann Whitney $p>0.5$ for all time points).

4.4 Activation of lymphocytes (CD69 expression) isolated from site of MT response.

We then decided to investigate whether the observed decrease in cell number present at the site of skin testing seen in the old was also correlated with a reduction in cellular activation. CD69 is expressed on a range of activated cells including B and T lymphocytes, macrophages and NK cells and is an early activation antigen. Upon activation, T lymphocytes produce an array of pro-inflammatory mediators, such as IFN-γ and TNF-α. These cytokines are critical for both the amplification of immune response in addition to inducing cutaneous vasodilatation and extravasation contributing to erythema and induration observed as part of the clinical response. A lack of activation of cells in the old could therefore explain reduction in the clinical score and reduced numbers of lymphocytes.

Lymphocytes isolated from blisters in the young and old were stained with antibodies immediately *ex vivo* for CD3, CD4 and CD69 and analysed by flow cytometry in order to establish whether the cells present in the blister fluid were activated (Figure 4.6). Analysis of CD69 expression on CD4+ T lymphocytes at Day 7 following the MT revealed a significant reduction in the percentage of CD4+ T lymphocytes expressing CD69 in the old compared with the young (Mann Whitney $p=0.0357$), indicating a reduction in cellular activation in the skin in the old (Figure 4.7). Levels of CD69 expression on lymphocytes isolated from blisters were significantly higher than in peripheral blood in both young and old ($p=0.01$ and $p= 0.001$ respectively).

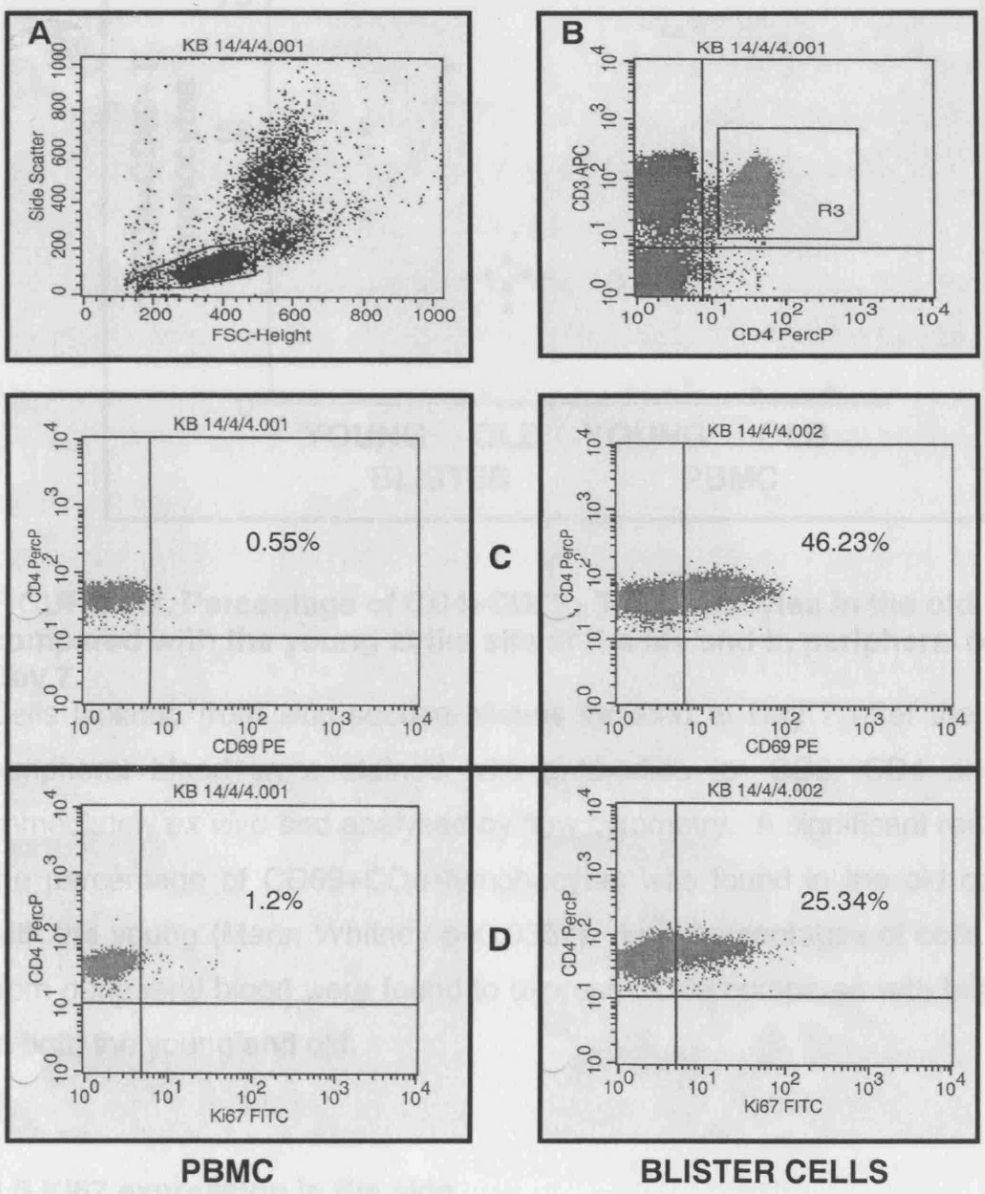


FIGURE 4.6 T lymphocyte intracellular Ki67 and CD69 expression in skin suction blister cells and PBMCs collected at day 7 after the Mantoux test following culture with PPD antigen.

Skin suction blister cells and PBMCs were stained immediately *ex-vivo* with CD3 APC, CD4 PercP, Ki67 FITC and CD69 PE. Live lymphocytes were gated on the basis of the FSC/SSC profile in order to exclude cell debris (A) and then gated on the basis of CD4+CD3+ expression in the blood (B) and blister (not shown) cell populations. Dot plots show the expression of CD69+ (C) and Ki67+(D) CD4+ cells from the PBMC and skin suction blister cells. Numbers in the upper right quadrants indicate the percentage of CD4+ Ki67+ or CD4+ CD69+ cells. The cell samples shown were from a representative young individual at Day 7 with a good clinical response following the injection of PPD into the skin.

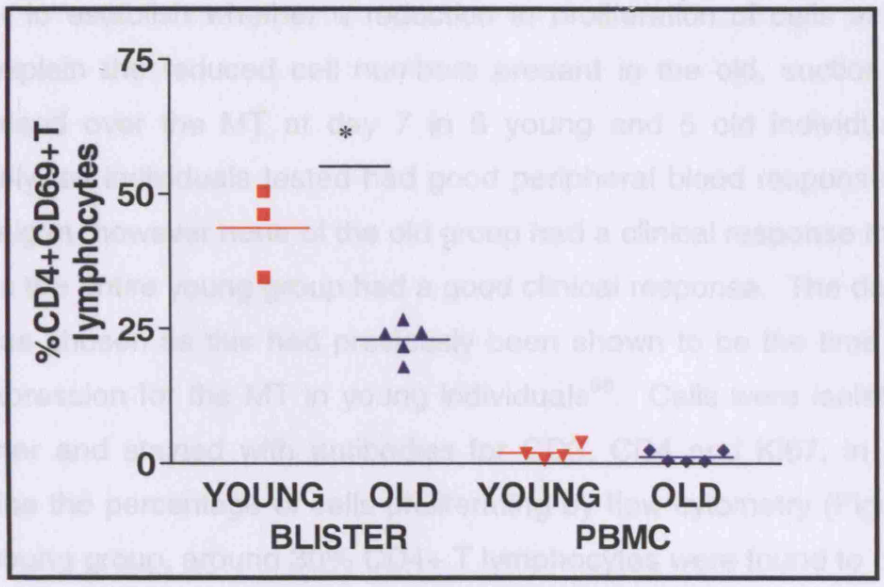


FIGURE 4.7. Percentage of CD4+CD69+ T lymphocytes in the old compared with the young at the site of the MT and in peripheral blood at Day 7.

Cells isolated from skin suction blisters induced at Day 7 after the MT and peripheral blood were stained with antibodies for CD3, CD4 and CD69 immediately *ex vivo* and analysed by flow cytometry. A significant reduction in the percentage of CD69+CD4+lymphocytes was found in the old compared with the young (Mann Whitney p=0.0357). Low percentages of cells isolated from peripheral blood were found to express CD69 compared with blister cells in both the young and old.

4.5 Ki67 expression in the skin

4.5.1 Proliferation of lymphocytes isolated from skin suction blisters at the site of the Mantoux Test (Ki67 expression)

Previously it has been proposed that cells proliferate locally at the site of the response to the MT in the skin in humans, accounting for increasing cell numbers observed rather than ongoing recruitment of T cells to the site from the blood. Reduced proliferation of CD4+ T lymphocytes within the skin in the old may explain the observed reduced numbers of lymphocytes present. We therefore investigated whether the cells present at the site of testing were proliferating using Ki67. Ki67 is a marker of cell proliferation that is expressed in the G1, S, G2 and M phases of the cell cycle.

In order to establish whether a reduction in proliferation of cells at the site could explain the reduced cell numbers present in the old, suction blisters were raised over the MT at day 7 in 5 young and 5 old individuals. As previously, all individuals tested had good peripheral blood responses to the PPD antigen, however none of the old group had a clinical response to the MT whereas the entire young group had a good clinical response. The day 7 time point was chosen as this had previously been shown to be the time of peak Ki67 expression for the MT in young individuals[88]. Cells were isolated from the blister and stained with antibodies for CD3, CD4 and Ki67, in order to determine the percentage of cells proliferating by flow cytometry (Figure 4.7). In the young group, around 30% CD4+ T lymphocytes were found to be in cell cycle as indicated by Ki67 expression. (mean: 28.01% SD±14.81) (Figure 4.8). Minimal blood CD4+ and CD8+ T lymphocyte proliferation occurred during the course of the MT. In addition, only small numbers (<2%) of CD8+ SB T cells expressed Ki67. This confirmed previous findings (unpublished data, Reed JR). In the old group, mean Ki67 levels were reduced at Day 7 compared with the young however this did not reach statistical significance (Mann Whitney p=0.095) (Figure 4.8A).

In order to determine whether proliferation in the skin could be induced by the trauma of injection alone, 0.1ml of Mantoux control solution was injected in to the forearm skin in 4 old and 3 young volunteers (Figure 4.8B). The Mantoux control solution was obtained from the same suppliers as the Mantoux test solution and contained the diluent of the tuberculin purified protein derivative only. Suction blisters were induced at Day 7 and isolated skin suction blister cells were stained as for the MT SB cells. Ki67+ CD4+ T cells were identified in skin suction blister cells isolated from the site of the injection of control solution. Although in the young group there was a reduction in the percentage of proliferating cells following the injection of the control solution compared with the MT (Wilcoxon ranked pairs test p=0.04), no difference was found between the MT or control skin tests in the old group (Mann Whitney test p=0.21). This implies that the level of Ki67 expression seen in old PPD

blisters is probably representative of that induced by trauma rather than due to an antigen-specific response to the PPD antigen (Figure 4.8).

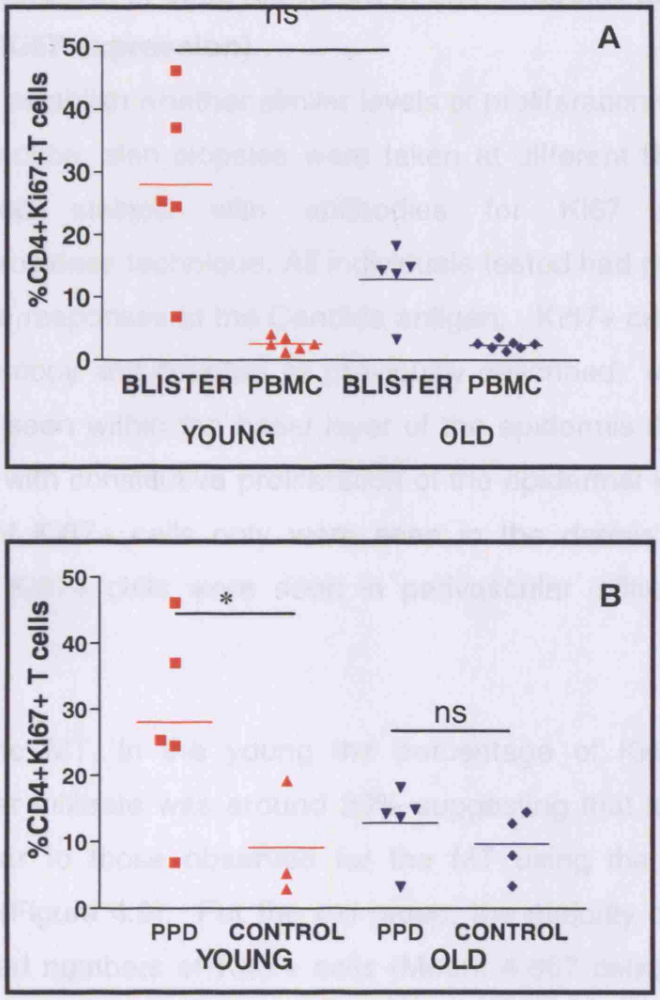


FIGURE 4.8 Percentage CD4+ Ki67+ lymphocytes in peripheral blood and SB at Day 7 after the MT and injection of the Mantoux control solution.

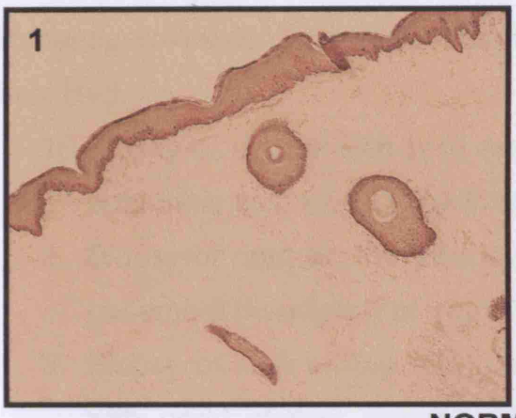
A. Skin suction blisters were induced at Day 7 after the MT in young and old volunteers. Suction blister cells and PBMCs were isolated and stained with antibodies for CD4 and Ki67 in order to identify CD4+ Ki67+ T lymphocytes by flow cytometry (Figure 4.7). A reduction in the mean number of Ki67+ CD4+ T lymphocytes was seen in the old compared with the young, although this did not reach statistical significance (Mann Whitney test p=0.095).

B. Skin suction blisters were also induced at Day 7 at the site of injection of Mantoux control test solution in the young and old. SB cells were isolated and stained as for the cells in Figure 5a. In the young, a reduction in the expression of Ki67 on CD4+ T lymphocytes was seen at the site of control solution injection compared with the MT test site (p=0.04). However, in the old, no significant difference in the expression of Ki67 was found between the MT test site and the control test site (p>0.5).

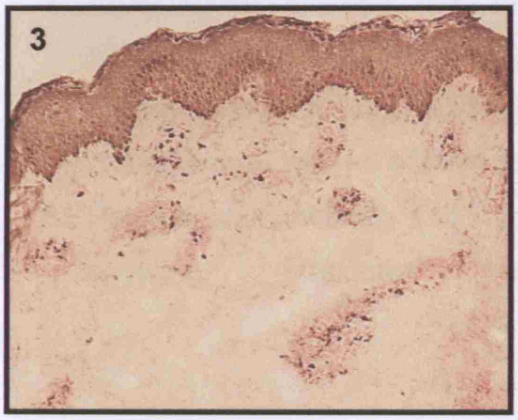
4.5.2 Proliferation of cells identified in skin biopsies at site of Candin skin test (Ki67 expression)

In order to establish whether similar levels of proliferation were found following Candin injection, skin biopsies were taken at different time points after skin testing and stained with antibodies for Ki67 using an indirect immunoperoxidase technique. All individuals tested had good peripheral blood proliferative responses to the Candida antigen. Ki67+ cells were identified by light microscopy and counted as previously described. In all samples, Ki67+ cells were seen within the basal layer of the epidermis (Photo plate 2 (1+2)) consistent with constitutive proliferation of the epidermal keratinocytes. Small numbers of Ki67+ cells only were seen in the dermis up to day 7 when numerous Ki67+ cells were seen in perivascular infiltrates (Photo plate 2 (2+3)).

As with the MT, in the young the percentage of Ki67+ cells within the perivascular infiltrate was around 20% suggesting that levels of proliferation were similar to those observed for the MT using the skin suction blister technique (Figure 4.9). For the old group, the majority of the old individuals had reduced numbers of Ki67+ cells (Mean: 4.867 cells) compared with the young (Mean: 20.92 cells)(Figure 4.9) although this did not reach statistical significance (Mann Whitney p=0.06). One old individual was found to have markedly higher numbers of cells expressing Ki67 (circled Figure 4.9A) compared with the rest of the old group. This old individual had a good clinical score in the skin (clinical score =6), in addition to high numbers of infiltrating perivascular T cells present at day 7 (mean number perivascular CD4+ T cells: 80.8 cells vs overall mean for old group: 33.24), indicating that this individual was not representative of the old population as a whole. Correlation of clinical score at Day 3 with the number of Ki67+ cells in both young and old groups however showed a positive correlation with increasing numbers of Ki67 cells with increasing clinical score (Spearman r=0.863, p=0.0045). It therefore seems likely that there is reduced Ki67 expression in the old with absent or poor clinical responses to the Candin skin test.



NORMAL SKIN



YOUNG SKIN DAY 7

OLD SKIN DAY 7

PHOTO PLATE 2

PHOTO PLATE 2. Ki67+ cells in the skin following Candin skin testing.
Fresh frozen skin biopsies were stained with Ki67 using an indirect immunoperoxidase technique. Sections were counterstained with Nuclear Fast Red.

1. Biopsy of normal skin (x10 magnification). Showing Ki67+ cells within epidermis and around hair follicles.
2. Biopsy of normal skin (x40 magnification). Showing Ki67+ cells along basement membrane of epidermis
3. Biopsy of skin at day 7 following Candin injection in young individual with good clinical response showing Ki67+ cells within perivascular infiltrates.
4. Perivascular infiltrate (x40 magnification) from biopsy shown in 3 showing Ki67+ cells (arrowed).
5. Biopsy of skin at day 7 (x10 magnification) following Candin injection in old individual with no clinical response to Candin injection.
6. Perivascular infiltrate (x40 magnification) from biopsy shown in 5 showing reduced numbers of Ki67+ cells.

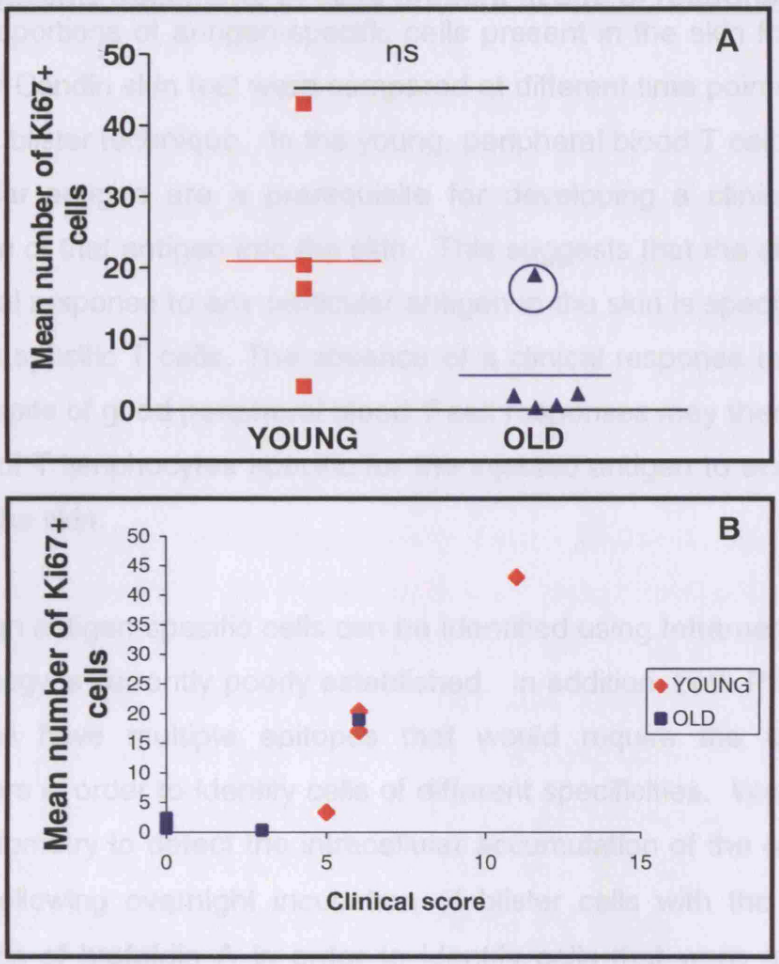


FIGURE 4.9 Numbers of Ki67 cells in perivascular infiltrates in young compared with old at Day 7.

Skin biopsies were taken at Day 7 following the injection of the Candin skin test in the young and old. Biopsies were stained using antibodies for Ki67 and the indirect immunoperoxidase technique. Ki67+ cells were seen predominantly within the perivascular infiltrates. Ki67+ cells within frames centred over the 5 largest perivascular infiltrates per section were counted and the mean number of Ki67+ cells per frame per individual was calculated.

A. There was a reduction in the mean number of Ki67 cells in the old)compared with the young although this did not reach statistical significance (Mann Whitney test p=0.06). One old individual had a relatively large number of Ki67+ cells (circled), however they had a good clinical and lymphocytic response to the Candin skin test and were therefore not representative of the old group as a whole.

B. The number of Ki67+ cells at Day 7 as calculated in Figure 6a was correlated with the clinical score seen at Day 3 (the peak of the clinical response) in the young and old. This showed a strong positive correlation between clinical response and the number of Ki67+ cells counted in the perivascular infiltrates (Spearman r=0.863, p=0.0045).

4.6. Antigenic specificity of cells present at site of response in skin

The proportions of antigen-specific cells present in the skin following the MT and the Candin skin test were compared at different time points using the skin suction blister technique. In the young, peripheral blood T cell responses to a particular antigen are a prerequisite for developing a clinical response to injection of that antigen into the skin. This suggests that the ability to develop a clinical response to any particular antigen in the skin is specific and involves antigen specific T cells. The absence of a clinical response in the skin in the old, in spite of good peripheral blood T cell responses may therefore be due to failure of T lymphocytes specific for the injected antigen to accumulate at the site in the skin.

Although antigen-specific cells can be identified using tetramers, MHC class II technology is currently poorly established. In addition, both PPD and candida antigens have multiple epitopes that would require the use of multiple tetramers in order to identify cells of different specificities. We therefore used flow cytometry to detect the intracellular accumulation of the effector cytokine IFN-γ following overnight incubation of blister cells with the antigen in the presence of brefeldin A in order to identify cells that were antigen specific. Skin suction blister cells contain a population of antigen- presenting cells and therefore the addition of antigen alone is sufficient to allow for antigen presentation to specific T lymphocytes. This was confirmed by previous experiments that demonstrated that the addition of additional APCs as irradiated autologous PBMCs to the skin suction blister cells did not augment IFN-γ production (unpublished work, Reed JR).

4.6.1 Antigenic specificity of skin suction blister T lymphocytes isolated at the site of the MT in young and old.

Initially, skin suction blister cells from blisters induced at days 7, 15 and 21 after the MT were assessed for antigenic specificity in the young group (Figure 4.11). Earlier time points were not investigated for the MT as previous work had shown that only very small percentages of antigen specific T lymphocytes are present before 7 days [88]. In this experiment, in the young, the percentages of antigen specific, IFN-γ producing, SB CD4+ T lymphocytes increased during the course of the MT response with a peak in the percentage of antigen specific cells at day 15, confirming previous findings. An increase in antigen specific cells was seen from Day 7 to day 15 (mean 15 to 30 % respectively) and then a reduction at day 21 (mean 26%) (Figure 4.10 A). At all time points the percentage of antigen specific T cells in the blood remained low (<1.4%) and relatively constant (Kruskall Wallis test p=0.09) and was significantly lower than the percentages of antigen specific cells isolated from the skin (Figure 4.10A). Background IFN-γ expression in CD4+ cells in the unstimulated blister control samples was also low and remained constant during the course of the MT (range of means 0-0.64%).

Previously it has been demonstrated that stimulation of skin blister cells isolated from the site of a MT with tetanus toxoid resulted in minimal IFN-γ expression (<1%), indicating that the accumulating T cells in the skin are specific for the injected antigen. As an additional control, 2 young individuals had paired suction blisters on opposite forearms, one with Mantoux control solution and the other with the MT. In both individuals a small percentage of cells specific for antigen were recruited in to the skin at the site of Mantoux control solution injection (MTvs Mantoux control: 32.4%vs6.62% and 25.51%vs2.81%), indicating that non-specific recruitment of antigen-specific T cells can occur at sites of trauma in the skin. This is advantageous as it may allow for T cells with a variety of antigenic specificities to enter wounds that are potentially infected with a variety of pathogens.

In the old, a dramatic reduction in the number of antigen-specific cells within the SB was seen for all time points compared with the young (Mann Whitney Day 7 p=0.0013, Day 15 p=0.0028, Day 21 p=0.001) (Figure 4.10B). Although a slight increase in the mean number of antigen specific cells between day 7 and 15 was seen in the old, this did not reach statistical significance (Wilcoxon ranked pairs test p=0.2). There was no significant difference between the number of antigen specific cells in the blood and blister for all time points. In contrast to the young group, in the old group there is no expansion of the antigen specific T cell population during the course of the MT response and this may account for the observed reduction in clinical response in addition to reduced numbers of lymphocytes present.

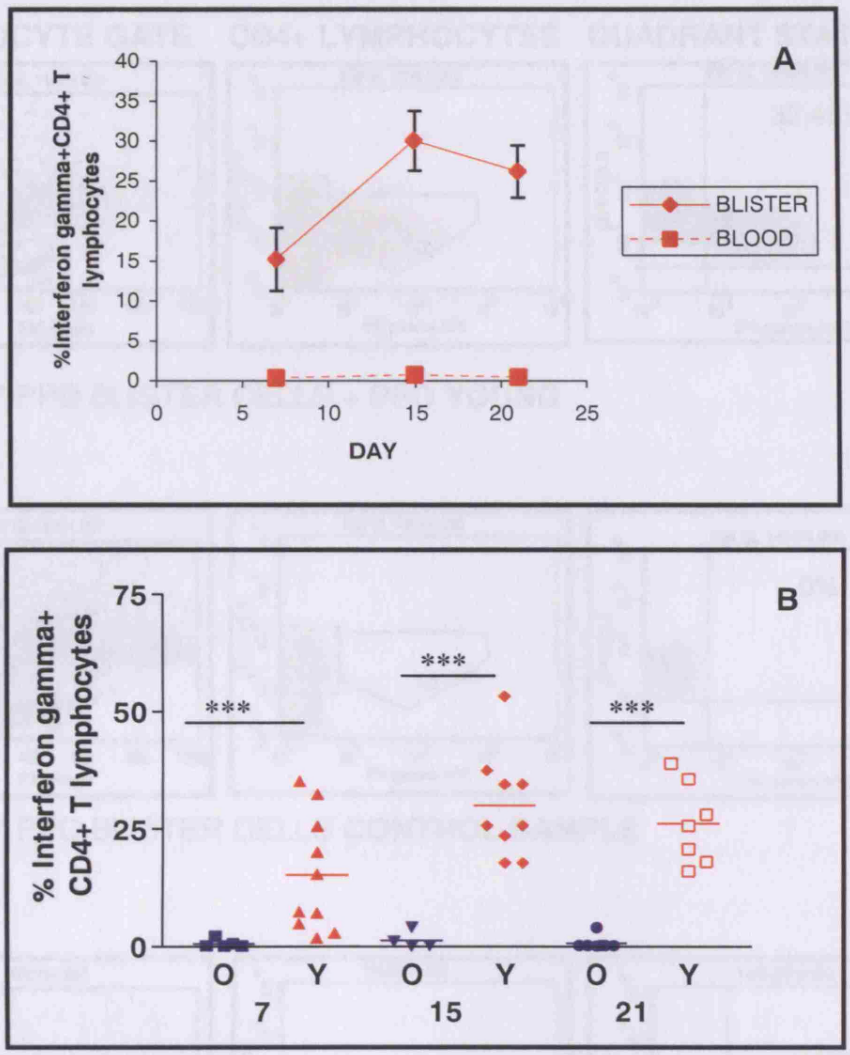


FIGURE 4.10 The kinetics of PPD-specific T cell infiltration in the skin following the MT in young and old.

The percentage of candida specific CD4+ T cells in the peripheral blood and skin was determined in the old (O) and young (Y) by examining for intracellular cytokine staining using flow cytometry (Figure 4.11). Skin blister and PBMC were stimulated with PPD antigen overnight in the presence of brefeldin A. Unstimulated controls were also performed. Live CD3+CD4+ cells were gated and at least 10,000 CD4+T cell events were acquired. **A.** The percentage of CD4+ T lymphocytes positive for IFN-γ expression following the MT in skin blister cells and peripheral blood in the young was determined. The data points show the mean ±SEM of 7-10 experiments performed at each time point. A peak in the percentage of PPD- specific cells in the blister was found at day 15. At all time points measured the percentage of antigen specific (CD4+ IFN-γ) cells was significantly higher in the SB cell population compared with the blood.

B. There was a significant reduction in the percentage of antigen- specific slom blister cells in the old compared with the young for all measured time-points after the MT (Mann Whitney Day 7 p=0.0013, Day 15 p=0.0028, Day 21 p=0.001)

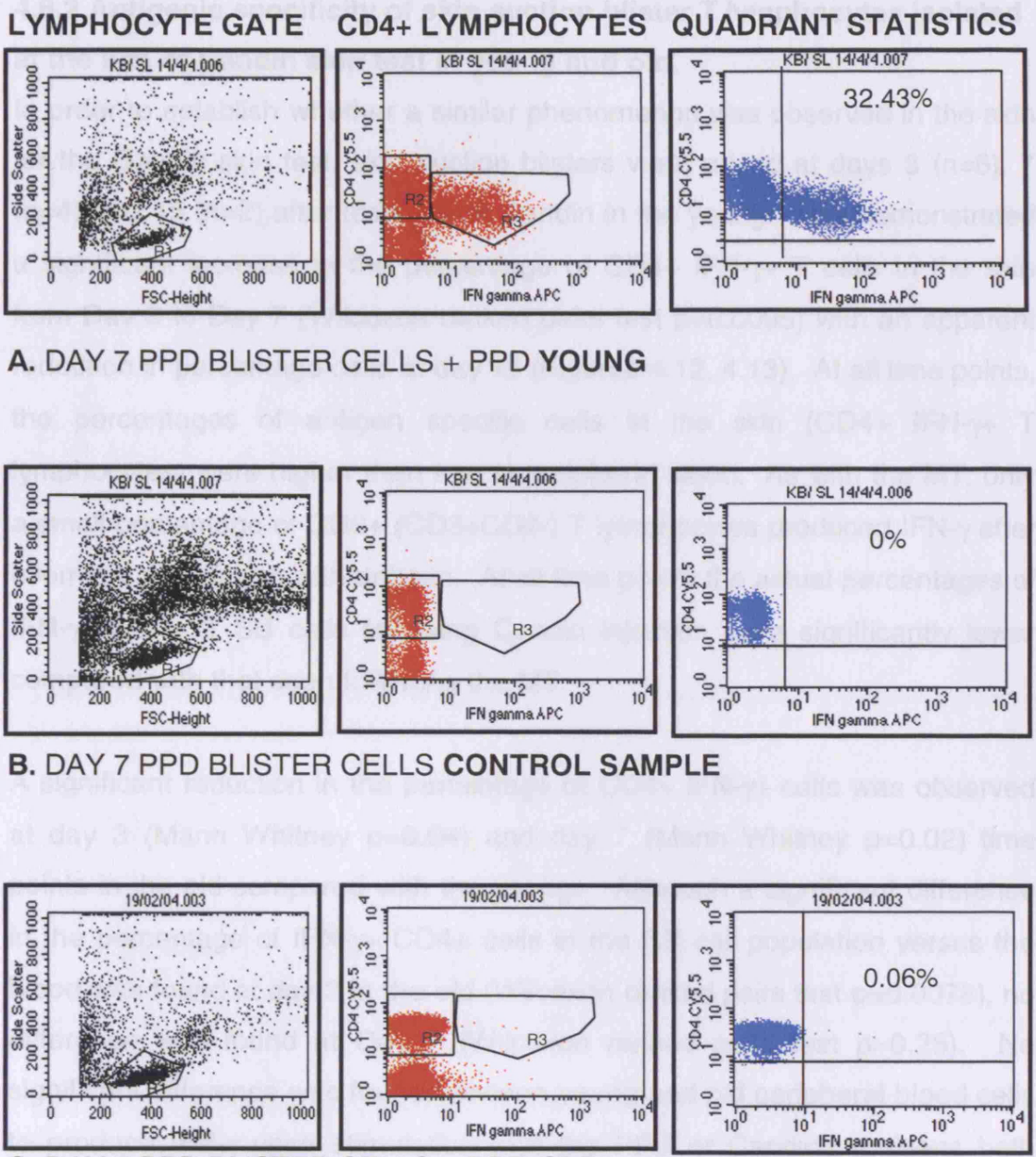


FIGURE 4.11 T lymphocyte intracellular IFN-γ expression in day 7 Mantoux test blister cells in young, old and control samples.
Skin suction blister cells from the site of PPD injection were incubated for 15 hours with or without PPD in the presence of Brefeldin A, which was added after the first 2 hours of culture. The cells were stained with CD4 Cy5.5 and IFN-γ APC. Live lymphocytes were identified and gated on the basis of the FSC/SSC profile in order to exclude cell debris. Representative dot plots show the expression of IFN-γ in CD4+ cells in **A.** skin suction blister cells from a young individual with a good clinical response following PPD stimulation *in vitro,* **B**. in skin suction blister cells isolated from the site of the response without PPD stimulation *in vitro* and **C.** skin suction blister cells isolated from the site of PPD injection from an old individual without a clinical response. The figure in the right upper quadrant of the quadrant statistics column represents the percentage of CD4+ T cells positive for IFN-γ expression.

4.6.2 Antigenic specificity of skin suction blister T lymphocytes isolated at the site of Candin skin test in young and old.

In order to establish whether a similar phenomenon was observed in the skin for the Candin skin test, skin suction blisters were raised at days 3 (n=6), 7 (n=4) and 15 (n=2) after testing with Candin in the young. This demonstrated a significant increase in the percentage of CD4+ IFN-γ+ T cells in the skin from Day 3 to Day 7 (Wilcoxon ranked pairs test p=0.0095) with an apparent reduction in percentage cells at day 15 (Figures 4.12, 4.13). At all time points, the percentages of antigen specific cells in the skin (CD4+ IFN-γ+ T lymphocytes) were higher than that in peripheral blood. As with the MT, only a small percentage of CD8+ (CD3+CD4-) T lymphocytes produced IFN-γ after overnight stimulation with antigen. At all time points the actual percentages of IFN-γ+ CD4+ T SB cells following Candin injection were significantly lower compared with that seen following the MT.

A significant reduction in the percentage of CD4+ IFN-γ+ cells was observed at day 3 (Mann Whitney p=0.04) and day 7 (Mann Whitney p=0.02) time points in the old compared with the young. Although a significant difference in the percentage of IFN-γ+ CD4+ cells in the SB cell population versus the blood was found at day 3 in the old (Wilcoxon ranked pairs test p=0.0078), no difference was found at Day 7 (Wilcoxon ranked pairs test p=0.25). No significant difference was found between young and old peripheral blood cells to produce IFN-γ upon stimulation with the PPD or Candida antigens both prior to skin testing and after skin testing for the day 3, 7 and 14 time points (data not shown).

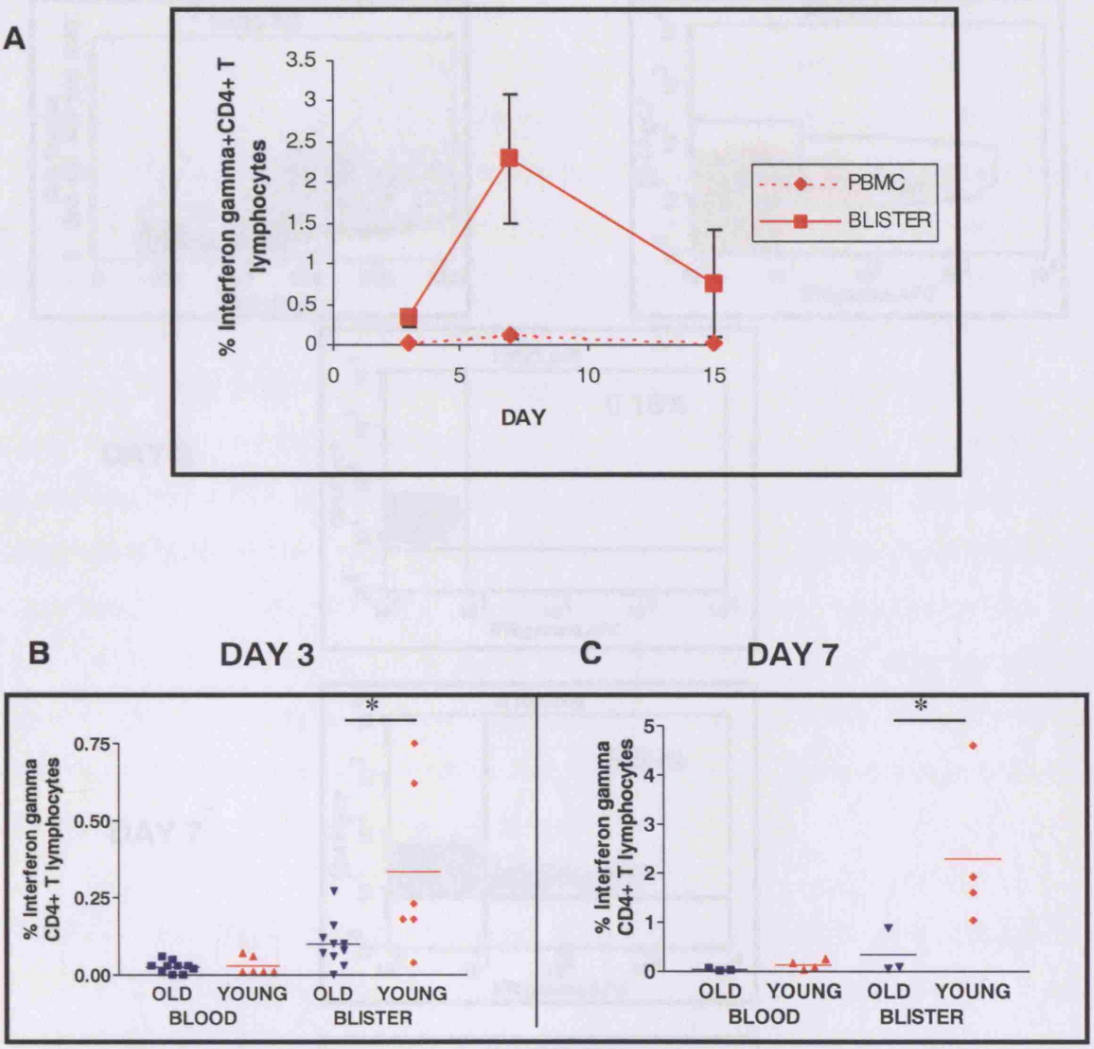


FIGURE 4.12 Kinetics of candida-specific T cell infiltration in the skin following Candin skin testing in young and old.

The percentage of candida specific CD4+ T cells in the peripheral blood and skin was determined by examining for intracellular cytokine staining using flow cytometry (Figure 4.13). Skin suction blister cells and PBMCs were stimulated with Candida antigen overnight. Unstimulated controls were also performed. Live CD3+CD4+ cells were gated. At least 10,000 CD4+T cell events were acquired. A peak in the percentage of IFN-γ+ CD4+ T lymphocytes was seen at Day 7 in the young **(A),** with percentages of cells significantly higher than PBMCs at all time points. The data points in A show the mean ±SEM of 2-5 experiments performed at each time point. A significant reduction in the percentages of candida specific (CD4+IFN-γ+) SB cells was seen at Day 3 **(B)** (Mann Whitney p=0.04 and Day 7 **(C)** (Mann Whitney p=0.02) in the old compared with the young.

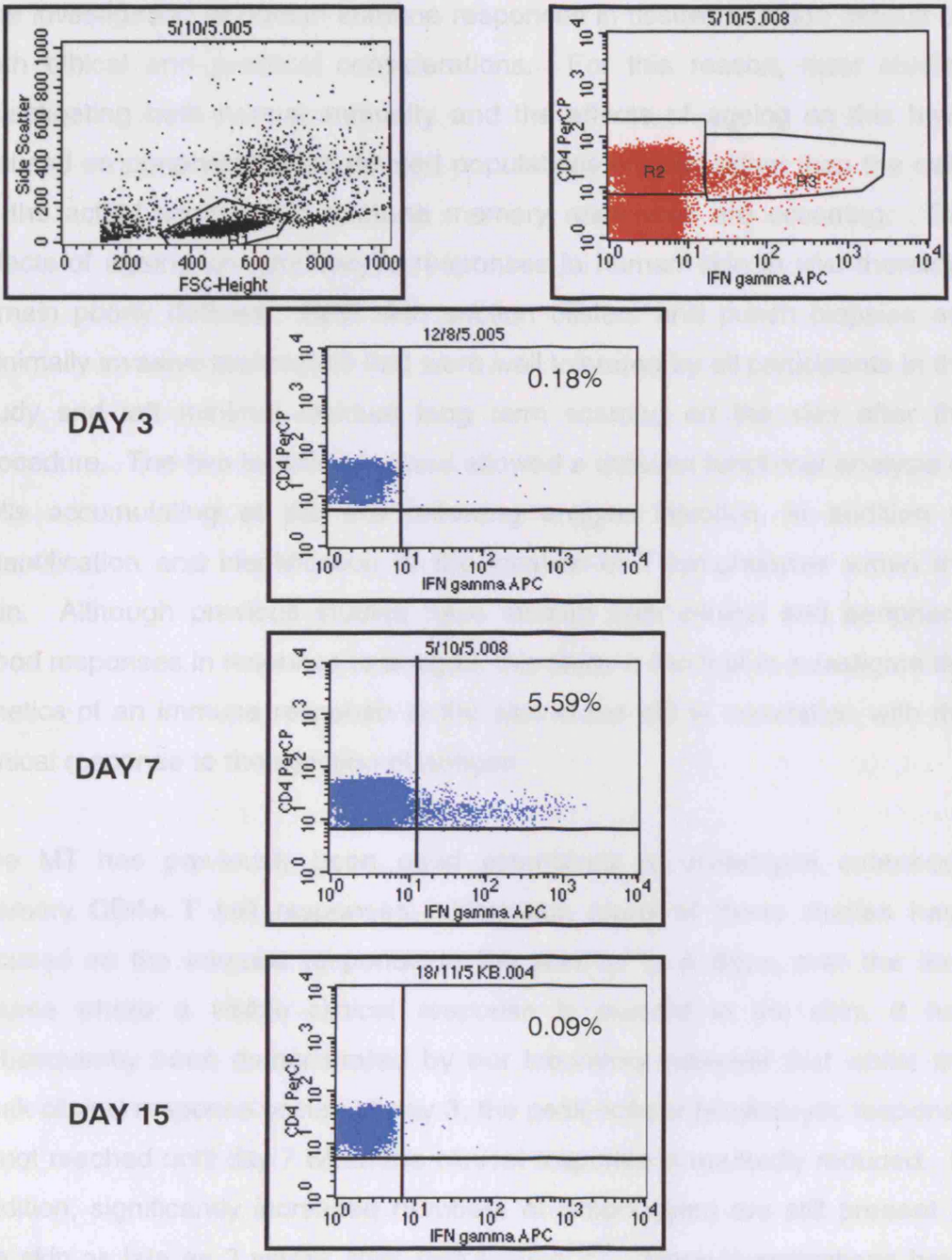


FIGURE 4.13 T lymphocyte intracellular IFN-γ expression in day 3, 7 and 15 Candin skin test blister cells in young individuals.

Skin suction blister cells from the site of Candin injection at 3, 7 and 15 days after injection were incubated for 20 hours with candida antigen in the presence of Brefeldin A, which was added after the first 15 hours of culture. The cells were stained with CD4 PerCP and IFN-γ APC. Live lymphocytes were identified and gated on the basis of the FSC/SSC profile in order to exclude cell debris. Dot plots show expression of IFN-γ in CD4+ cells (R2 and R3 gates) from skin suction blister cells collected from representative young volunteers at the measured time points. The percentage of CD4+ IFN-γ+ cells is shown in the upper left quadrant.

4.7 Discussion

The investigation of human immune responses in tissues is made difficult by both ethical and practical considerations. For this reason, most studies investigating both normal immunity and the effects of ageing on this have focused on peripheral blood-derived populations of cells rather than the cells at the actual sites where immune memory responses are occurring. The effects of ageing on lymphocytic responses in human skin *in vivo* therefore remain poorly defined. Both skin suction blisters and punch biopsies are minimally invasive techniques that were well tolerated by all participants in the study and left minimal residual long term scarring on the skin after the procedure. The two techniques used allowed a detailed functional analysis of cells accumulating at the site following antigen injection, in addition to quantification and identification of the location of T lymphocytes within the skin. Although previous studies have studied both clinical and peripheral blood responses in response to antigen, this study is the first to investigate the kinetics of an immune response in the skin in the old in correlation with the clinical response to the injection of antigen.

The MT has previously been used extensively to investigate cutaneous memory CD4+ T cell responses. However, many of these studies have focused on the immune response in the skin up to 4 days, over the time course where a visible clinical response is present in the skin. It has subsequently been demonstrated by our laboratory however that whilst the peak clinical response occurs at day 3, the peak cellular lymphocytic response is not reached until day 7 when the clinical response is markedly reduced. In addition, significantly increased numbers of lymphocytes are still present in the skin as late as 3 weeks after skin testing[64;88]. Many investigations have therefore significantly underestimated the role of lymphocytes in DTH responses in the skin.

In view of the dissociation between clinical and cellular responses seen in the young the aim of this study was to establish whether the reduced clinical response in the skin in the old was truly reflective of reduced lymphocytic responses at the site of the response. A reduction in the numbers of

lymphocytes was seen for the MT response at day 7 in the old group when peak numbers of lymphocytes are seen in the skin in the young. A reduction in cell numbers was also seen at 1, 3 and 7 days after skin testing with Candin. Importantly, a positive correlation between clinical response and numbers of cells within perivascular infiltrates for both young and old individuals was demonstrated with reduced numbers of lymphocytes in all individuals who failed to develop a clinical response to the injection of antigen. This data is important as it further indicates that the clinical DTH responses in the skin are dependent on T lymphocyte responses at the site. We have however only studied lymphocytic responses at a set number of time points. It remains possible that lymphocyte numbers peak after day 7 in the old due to a slower response time. In the future it would therefore be advantageous to study all phases of the response, in particular at later time points.

Although the majority of studies would suggest that there is no age related decrease in the total number of CD3+ T lymphocytes in old humans or mice, some studies have indicated that this is the case. The age related decline in the number of T cells present in the skin during the response could therefore be related to an overall decline in the circulating number of T cells in the old. In our study, however, there was no evidence that any of the volunteers in the old age group had reduced cell counts in peripheral blood based on PBMC counts isolated from whole blood. The overall phenotype of T lymphocytes is also thought to change with ageing with a shift of cells with a naïve phenotype (CD45RA+, CD45RO-) to a memory phenotype (CD45RA-CD45RO+)[191;192]. Previously our laboratory has established that, in the young, the predominant CD4+ T cell phenotype during the course of the response is CD45RA-, CD45RO+. The older group was therefore likely to have had increased circulating numbers of lymphocytes with a memory phenotype compared with the young group. However, a reduction in memory T cell receptor repertoire also occurs with ageing[174]. The presence of peripheral blood T lymphocyte responses in the old to both PPD and Candida suggests that the old population investigated in this study did have a population of cells within peripheral blood that had the specific T cell receptors for these antigens.

Previous studies on old humans have observed that there is an increase in the frequency of CD4+ T cells compared with CD8+ T cells in peripheral blood resulting in the increased CD4:CD8 ratio. It is therefore of interest that a reversal of this ratio was seen during the course of the response in the skin in the old, with a reduced CD4:CD8 ratio compared with the young at later time points. This observation suggests that although there is an initial recruitment of both CD8 and CD4+ T lymphocytes to the site of injection occurred in both young and old individuals, the overall increase in lymphocyte numbers during the DTH response seen in the young is dependent on the expansion of the CD4+ T lymphocyte population alone.

Reduced functional capacity of T lymphocytes present in the skin may be responsible for reduced cellular response. In particular, a lack of cellular proliferation at the site may be responsible for the reduced expansion of the CD4+ T lymphocyte population in the skin. Conventionally it is thought that, during a secondary immune memory response, memory T cells resident in the draining lymph node are activated and divide following interaction with antigen presenting cells that have migrated from the skin to the lymph node. These memory T cells are then recruited to the skin at the site of antigenic challenge. This model has been proposed based on data from murine studies that have shown that although antigen specific memory cells are present in both non-lymphoid and secondary lymphoid organs, they do not proliferate within non-lymphoid organs during primary or secondary immune responses[84;85]. However, published data from human studies has in fact indicated that a significant proportion of the expansion of lymphocyte numbers within the skin is mediated by localised T cell proliferation[88]. This model is further supported by the results from this study that show that there is a marked increase in the percentage of Ki67 positive T cells at day 7 in the skin, at the time when lymphocyte numbers reach their peak, without any detectable increase in Ki67 positive cells within the peripheral blood population.

Previous studies have indicated reduced peripheral blood cell proliferative responses to mitogens or antigens in ageing mice and humans[175-177]. In this study, however, we have not found any differences in the proliferative capacity

of peripheral blood cells to respond to either the PPD or Candida antigens (discussed in Chapter 3), suggesting that any observed reduction in the proliferative capacity of cells within the skin is related to either the population of cells recruited or the local microenvironment in the skin. We have demonstrated a small, but non-statistically significant, reduction in the percentage of Ki67 + CD4+ T lymphocytes present in the skin for both the MT and Candin skin test in the old compared with the young at all time points. Importantly, comparable levels of Ki67 expression in the CD4+ T lymphocyte population were seen in the old following the injection of control solution and the MT, suggesting that proliferation observed in the old is due to trauma rather than that driven by an antigen-specific response. Thus a lack of ability of antigen specific T cells to proliferate at the site would result in a failure of the CD4+ T cell population in the skin to expand appropriately with a resulting reduction in clinical and cellular response.

In this study we have demonstrated a marked reduction in the percentage of antigen-specific cells present in the skin during the Mantoux and Candin skin tests in the old compared with the young. The lack of antigenic specificity of cells present at the site in the old may also explain why there was a reduced level of T cell activation, as shown with a reduction in CD69 expression on suction blister cells. Antigen specific cells were identified on the basis of intracellular production of IFN-γ production after overnight stimulation with antigen. Both the MT and Candin skin test are thought to induce Th1 type lymphocytic responses in the skin with the production of predominantly IFN-γ. Some studies have shown a change in cytokine profiles after cellular activation in the old. It is therefore possible that cytokines other than IFN-γ, such as IL-2, may be produced by antigen specific cells in the old. However, in old mice lymphocytes have been shown to produce less IL-2 and more IFN-γ than T cells from young mice[131;186]. These results may have been biased by the larger numbers of memory T cells in the old mice as memory T cells are known to produce more interferon gamma than their naïve counterparts. Studies where individual cell phenotypes have been investigated have

subsequently shown no difference in cytokine production in young and old mice.

It is also possible that the observed reduction in antigen specific cells in the old may not be due to a reduction in antigen-specific cells per se but rather an inherent inability of these cells to produce IFN-γ. Although no difference in IFN-γ production was found for peripheral blood CD4+ T lymphocytes in the young and old groups, the skin microenvironment in the old could render T lymphocytes refractory to restimulation with antigen and also affect their ability to produce IFN-γ. It is also possible that the old skin suction blister cells may have contained reduced numbers or defective antigen presenting cells, required for restimulation of T cells by the antigen in our *in vitro* model. Further investigation, using autologous irradiated APCs from PBMCs added to the skin suction blister cells, would help to exclude absent or defective antigen presenting cells within the skin suction blister cell population as a reason for the apparent reduction in antigen specific cells.

Significantly lower percentages of antigen specific cells were identified during the course of the response to the Candin skin test compared with the MT in the young. Although this may be reflective of differing responses in the skin to different types of antigen, the reduced percentage of antigen specific cells was not reflective of a reduction in the clinical response to the Candin skin test compared with the MT. For both skin tests it was not possible to use the same reagent for *in vitro* work. This was thought to be attributable to the presence of preservative agents within the skin test that were toxic to the SB cells *in vitro.* It is therefore possible that the agent used to test for *in vitro* responses did not contain the full range of epitopes that were present in the skin test resulting in an underestimation of the actual number of antigen specific cells present. Previous studies have indicated that intracellular cytokine staining only detects between 30 to 90% of tetramer positive cells[296] and it is therefore possible that our data represents an underestimate of the percentage of antigen specific cells present in the skin. Of interest, other studies have found that less than 1% of cells infiltrating the skin are antigen specific[386]. The

variability in published data may be explained by differing skin tests and *in vitro* agents used to identify antigen specific cells.

It is of interest that at the peak of the clinical response there are very few antigen specific cells present at the site of the response. In spite of this, clinical responses are only seen in those who have antigen specific cells within the peripheral blood, suggesting that antigen specific T cells have to be involved early in the response. Clinical response in some individuals is seen after a matter of hours indicating that memory response is rapid following injection of antigen. It is possible that non-antigen specific mechanisms such as trauma or activation of innate pathway is sufficient to allow for the flux of a large number of T cells through the skin, of which only a very small number have to be antigen specific in order to mediate a response. Using this model, we have demonstrated the recruitment of antigen-specific cells to the sites of trauma alone in the skin in humans.

Another possibility is that an early, antigen specific response in the skin is mediated by memory T cells already resident within the skin. Large numbers of T cells are present in normal skin[65;66]. These cells have diverse T cell receptor repertoires, an effector memory phenotype[65] and are in close proximity with APC in the skin[79;80]. Thus, all components are present in the skin that should enable a rapid, antigen specific response to antigen in the skin. Although similar numbers of resident CD3, CD4 and CD8+ T cells were identified within both young and old skin in this study a functional defect in the resident lymphocyte skin cell population in the old could explain the observed reduction in both clinical and cellular responses to antigen injection in to the skin.

5. Skin homing receptor expression on peripheral blood lymphocytes and the production of cytokines and chemokines at the site of antigenic challenge.

5.1 Introduction

In Chapter 4 we demonstrated that there is a reduction in the number of lymphocytes at the site of the DTH response to both the MT and Candin skin test in the old compared with the young. In addition, we have shown that there are reduced cellular and clinical responses to the intradermal injection of a variety of antigens in the old in spite of the presence of antigen specific memory T cells within the peripheral blood. A possible explanation for the reduced number of lymphocytes present in the skin during the course of the DTH response, in spite of apparent immunity to the injected antigen in the peripheral blood compartment, is that in the old there is a defect in the recruitment of lymphocytes from the blood into the skin.

The migration of circulating T lymphocytes in to tissue- specific sites of inflammation is a complex process involving a range of adhesion molecules, cytokines, chemokines and the expression of their respective receptors on different cell types. In addition to the regulation of recruitment of cells to sites of inflammation, mechanisms are also in place to allow for routine immunosurveillance of non- inflamed tissues. Lymphocyte trafficking is a highly coordinated process and thus a breakdown of the underlying mechanisms involved may either result in inappropriate inflammation, as seen in inflammatory skin diseases such as psoriasis, or conversely immune suppression. The observation that the frequency of cutaneous infection[136] and neoplasia[149] increases with age could potentially be explained by defects

in lymphocytic recruitment in to the skin. In addition, atopic dermatitis (an inflammatory skin condition) improves with age[295] and this may again be explained by alterations in lymphocyte trafficking. Selective targeting of skin specific molecules involved in process of cellular migration and retention have become potential therapeutic targets for inflammatory disease.

Chemokines and cytokines produced during the course of the DTH may also play an important role not only in the recruitment of cells but also the retention of cells at the site, resulting in accumulation of cells. Recent evidence in humans suggests that the induction of chemokine receptors on CD4+ T cells following their entry in to inflamed tissue might play an important role in lymphocyte accumulation and also subsequent migration out of the skin resulting in resolution of the immune response.

5.2 Adhesion molecule expression on circulating T cells

The recruitment of lymphocytes into inflamed skin is dependent on the expression of a variety of cell surface receptors that enable lymphocyte interaction with the endothelium of cutaneous blood vessels and then subsequent transendothelial migration into the skin.

5.2.1 Cutaneous lymphocyte antigen expression on T lymphocytes in peripheral blood.

CLA expression on circulating T cells in the young and old was investigated in order to determine whether alterations in CLA expression could be responsible for the observed reduced lymphocytic response in the skin. Memory T lymphocytes localising to the skin express the cutaneous lymphocyte antigen (CLA) which functions as a ligand for E-selectin[110]. In inflamed skin, the expression of E-selectin on endothelium allows for transient reversible binding of endothelial cells with CLA+ lymphocytes from the peripheral blood, resulting in a process known as rolling where lymphocytes are seen to move in a linear direction along the endothelium. Upregulation of

expression of E-selectin is mediated by the pro-inflammatory cytokines IFN-γ and TNF-α[109].

PBMCs from young and old individuals were stained for CD3, CD4 and CLA and analysed using flow cytometry (Figure 5.3). No significant difference was identified between the groups for percentage CLA expression on total CD4+ T lymphocytes (Mann Whitney p=0.054) although a small increase in the expression of CLA on the total CD3+ lymphocyte population was seen in the old (Mann Whitney: p=0.01)(Figure 5.1A). CLA is expressed predominantly on memory rather than naïve T lymphocytes. In view of the higher proportions of memory versus naïve cell populations observed in the old it is possible that the higher proportions of memory T lymphocytes may compensate for an overall relative reduction in CLA expression which would not be identified by assessing CLA expression on the total T lymphocyte population. In view of this, CLA+ CD45RO+ memory CD4+ T lymphocytes were identified in the young and old groups using flow cytometry (Figure 5.2). No difference in CLA expression on CD4+CD45RO+ T lymphocytes was found between the young and old groups (Mann Whitney p=0.89) (Figure 5.1B).

CLA expression on antigen specific T lymphocytes in the young and old was also investigated. CLA is expressed only on T lymphocytes that have undergone primary activation in the draining lymph nodes in the skin[18]. It is therefore possible that different routes of initial exposure to the PPD antigen (ie via BCG vaccination in the young versus environmental exposure in the old) could result in changes in CLA expression on antigen-specific T lymphocyte expression of CLA in the young and old groups studied. PBMCs were cultured overnight in X-Vivo 15 medium with PPD antigen in the presence of brefeldin A. The cells were then stained for CD3, CD4, IFN-γ and CLA. As previously, antigen specific cells were identified on the basis of the intracellular production of IFN-γ after overnight stimulation with antigen. CLA expression was then compared for old and young groups on the antigen-specific (CD4+ IFN-γ +) T lymphocyte populations (Figure 5.3). No difference was found for CLA expression on PPD- specific cells in the young and old

(Mann Whitney p=0.89) (Figure 5.1C), demonstrating that percutaneous administration of PPD antigen in the young group did not result in increased CLA expression compared with the old who had not received vaccination.

CLA expression on CD4+ T cells specific for other antigens was also compared (Figure 5.1C). As for PPD, no significant difference was found for CLA expression on T lymphocytes specific for Candida and VZV in the young and old. It is of interest however that there was an overall reduction in the percentage of CLA positive antigen specific cells for the PPD compared with Candida antigens (Mann Whitney old p=0.01, young p=0.036)). This may reflect the fact that candida is a predominantly cutaneous pathogen whereas PPD is likely to be mucosal, respiratory pathogen.

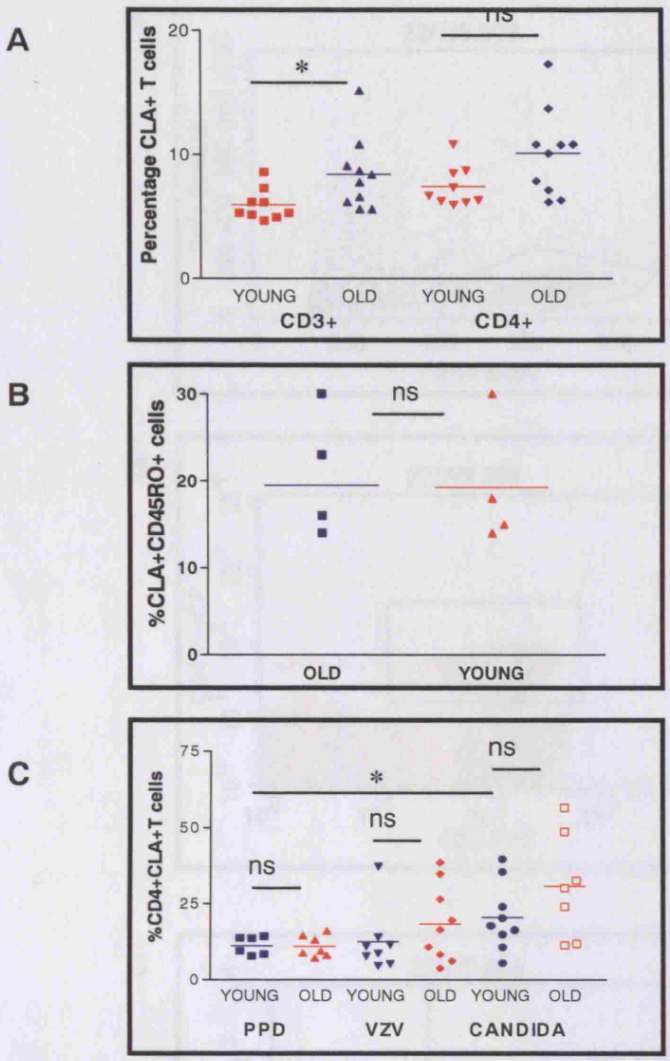


FIGURE 5.1 Expression of CLA on T lymphocytes in young and old: CD3 and CD4+ subsets, CD4+CD45RO subsets and CD4+ antigen specific cells.

PBMCs were collected from young and old were collected and the expression of CLA on different T lymphocyte subsets was determined using flow cytometry (Figure 5.3). The percentage of CLA+ lymphocytes within the total CD3+ and CD4+ lymphocyte populations was determined. No difference was found between the young and old for CD4+ lymphoctyes (Mann Whitney p=0.054) although a small increase in CLA expression on CD3+ lymphocytes was found in the old (Mann Whitney p=0.01) T lymphocyte subsets **(A).** The percentage of CLA+ lymphocytes within the CD4+CD45RO+ (memory T cell) population was also determined using flow cytometry (Figure 5.2). No difference was found between the young and old (Mann Whitney p=0.89) **(B).** The percentage of CLA+ cells within the PPD, VZV and Candida antigen-specific CD4+ T lymphocyte populations was determined **(C).** Antigen specific cells were identified by staining for IFN-γ production after overnight stimulation of PBMCs *in vitro* with antigen (Figure 5.3). No difference was found between the young and old groups, although higher percentages of CLA+ cells were present within the Candida compared with the PPD specific population (Mann Whitney old p=0.01, young p=0.036) **(C).**

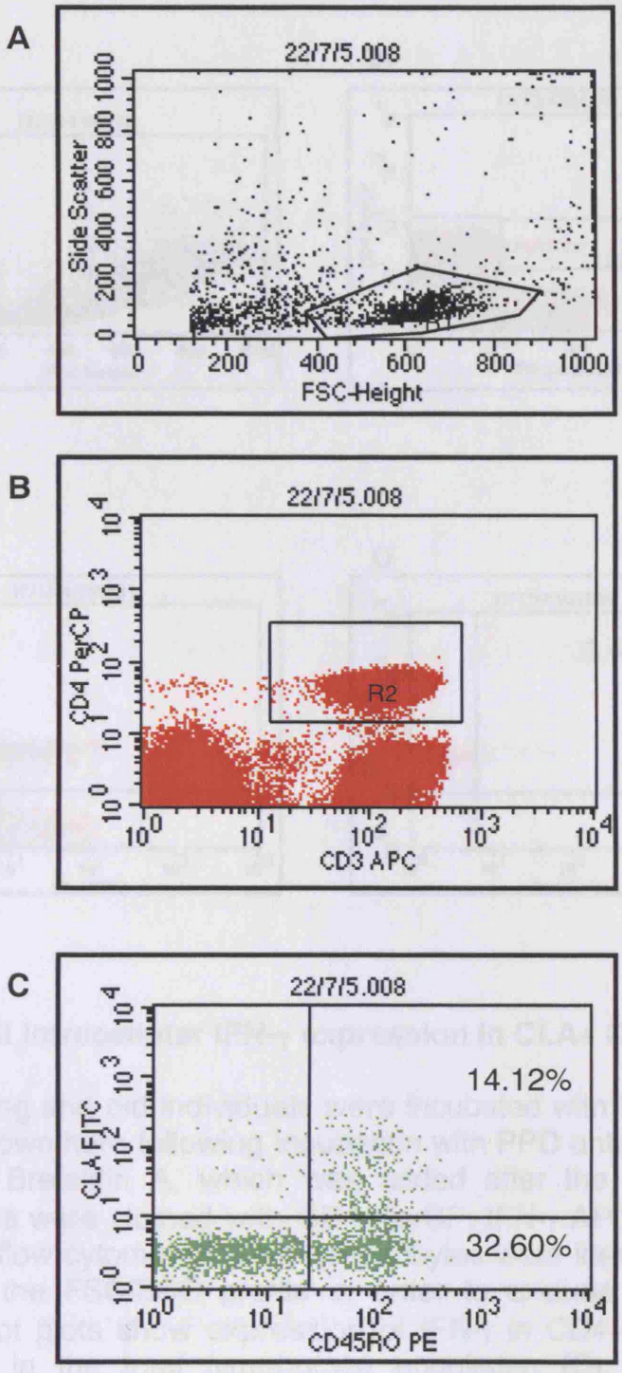


FIGURE 5.2 CLA expression on the CD4+CD45Ro+ T lymphocyte population.

PBMCs from young and old individuals were stained with CD3APC, CD4 PercP, and CD45RO PE and analysed by flow cytometry. Live lymphocytes were gated on the basis of the FSC/SSC profile in order to exclude cell debris **(A)** and then gated on the CD4+ population on the basis of CD4+CD3+ expression **(B).** The representative dot plot **C** shows the expression of CD45RO and CLA on the CD4+ lymphocyte population. The percentage of CLA+ cells within the CD45RO+ population was calculated using quadrant percentage statistics (30% in this individual).

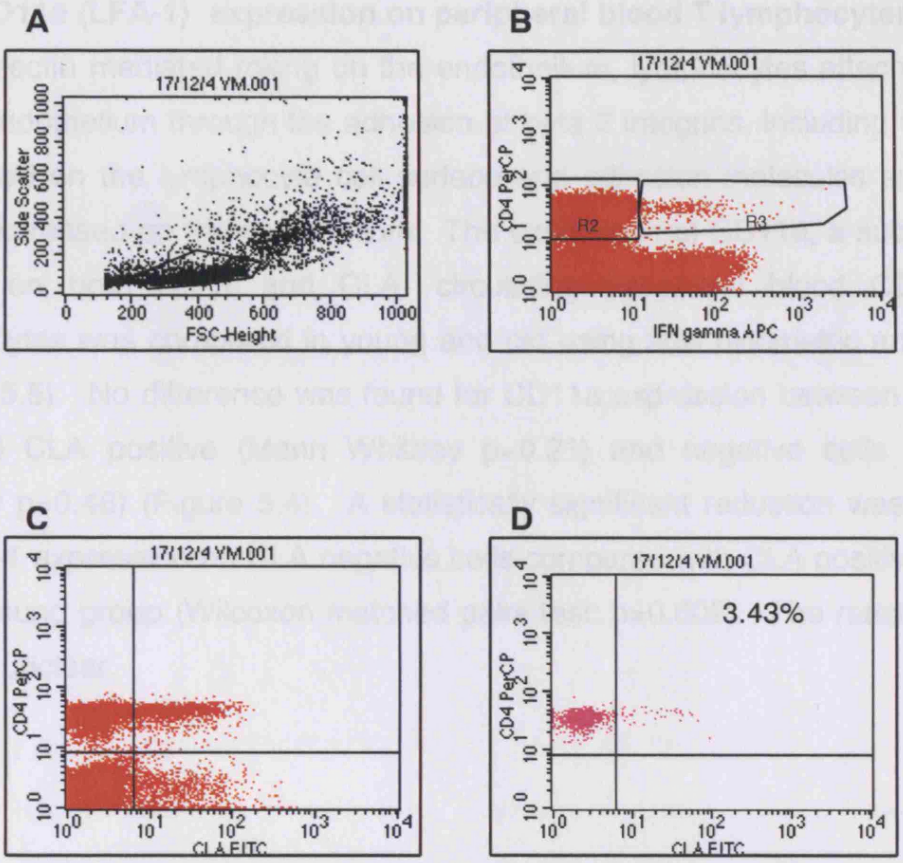


FIGURE 5.3 T cell intracellular IFN-γ expression in CLA+ CD4+ lymphocytes

PBMCs from young and old individuals were incubated with PPD, candida or VZV antigens (shown here following incubation with PPD antigen for 15 hours the presence of Brefeldin A, which was added after the first 2 hours of culture.) The cells were stained with CD4 PerCP, IFN-γ APC and CLA FITC and analysed by flow cytometry. Live lymphocytes were identified and gated on the basis of the FSC/SSC profile in order to exclude cell debris (**A**). Representative dot plots show expression of IFN-γ in CD4+ cells (gate R3) **(B)**, CLA+ cells in the total lymphocyte population **(C)**, and within the CD4+IFN-γ+ cell population **(D)** (gated on R3 shown in **B**). The percentage in the right upper quadrant in D represents the percentage of CLA+ antigen-specific CD4+ T lymphocytes within the peripheral blood.

5.2.2 CD11a (LFA-1) expression on peripheral blood T lymphocytes.

After selectin mediated rolling on the endothelium, lymphocytes attach firmly to the endothelium through the adhesion of beta 2 integrins, including LFA-1, expressed on the lymphocyte cell surface and adhesion molecules such as ICAM expressed on the endothelium. The expression of CD11a, a subunit of LFA-1, on both CLA+ and CLA- circulating peripheral blood CD4+ T lymphocytes was compared in young and old using flow cytometric analysis. (Figure 5.5). No difference was found for CD11a expression between young and old CLA positive (Mann Whitney p=0.21) and negative cells (Mann Whitney p=0.48) (Figure 5.4). A statistically significant reduction was found for LFA-1 expression on CLA negative cells compared with CLA positive cells in the young group (Wilcoxon matched pairs test: p=0.002). The reasons for this are unclear.

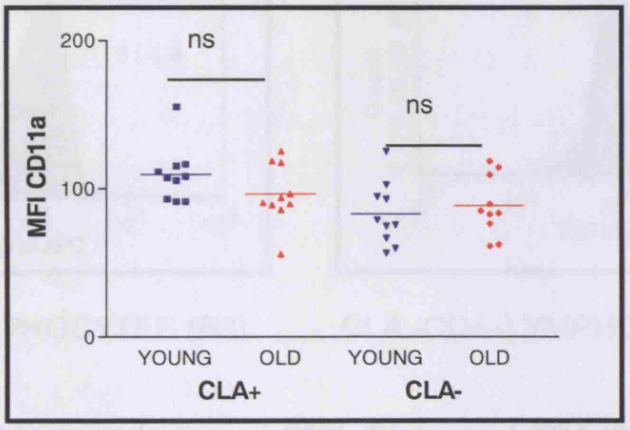
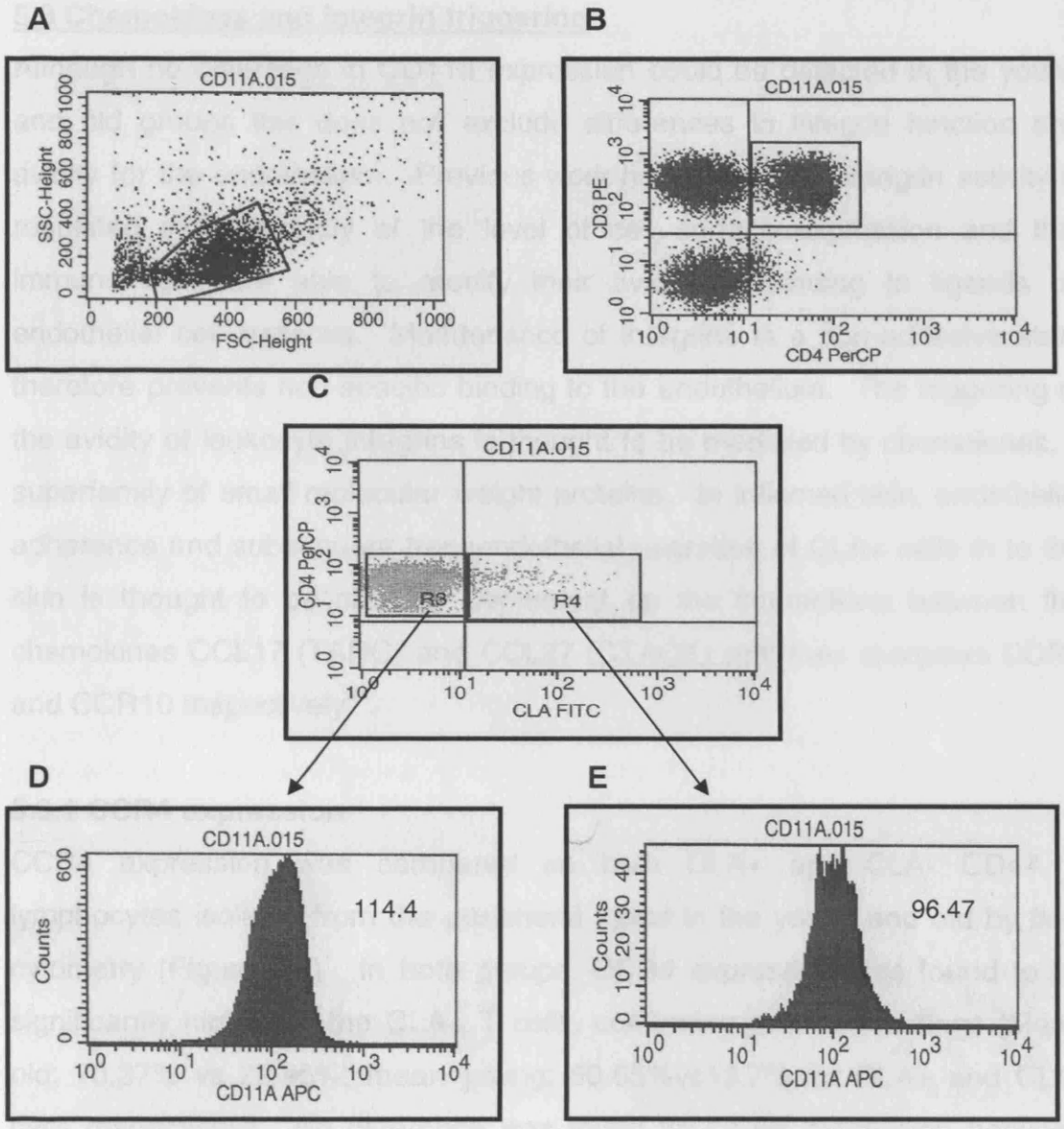


FIGURE 5.4 Expression of CD11a on CLA+ and CLA- CD4+ T lymphocytes in the young and old.

PBMCs isolated from young and old individuals were stained with antibodies for CD3, CD4, CLA and CD11a and analysed using flow cytometry (Figure 5.5). CD4+ lymphocytes were identified and the percentage of CD11a cells within the CD4+ lymphocyte population was calculated for CLA+ and CLA- populations. No significant difference was found for the percentage of CD11a+ cells within the CD4+CLA- (Mann Whitney p=0.21) or CD4+CLA+ cell populations (Mann Whitney p=0.48) for the old and young groups.



CLA- CD4+ LYMPHOCYTES (R3) CLA+CD4+LYMPHOCYTES (R4)

FIGURE 5.5 CD11a expression on CD4+CLA+ and CD4+CLA- T lymphocytes

PBMCs collected from young and old individuals were stained with anti- CD4 PercP, CD3PE, CLA FITC and CD11a APC. Live lymphocytes were identified and gated on the basis of the FSC/SSC profile in order to exclude cell debris (A). CD4+ cells were gated on on the basis of CD3+and CD4+ staining on the lymphocyte gate (B). CLA+CD4+ cells (R3 gate) and CLA-CD4+ cells (R4 gate) were identified (C). The median fluorescent intensity (MFI) was calculated for both CLA-CD4+ cells (R3 gate) (D) and CLA+CD4+ cells (R4 gate) (E). The figures on D and E are the calculated MFIs for the representative individual shown.

5.3 Chemokines and integrin triggering

Although no difference in CD11a expression could be detected in the young and old groups this does not exclude differences in integrin function and avidity for the endothelium. Previous work has shown that integrin activity is regulated independently of the level of cell surface expression and that immune cells are able to modify their avidity of binding to ligands on endothelial cell surfaces. Maintenance of integrins in a non-adhesive state therefore prevents non-specific binding to the endothelium. The triggering of the avidity of leukocyte integrins is thought to be mediated by chemokines, a superfamily of small molecular weight proteins. In inflamed skin, endothelial adherence and subsequent transendothelial migration of CLA+ cells in to the skin is thought to be critically dependent on the interactions between the chemokines CCL17 (TARC) and CCL27 (CTACK) with their receptors CCR4 and CCR10 respectively[87].

5.3.1 CCR4 expression

CCR4 expression was compared on both CLA+ and CLA- CD4+ T lymphocytes isolated from the peripheral blood in the young and old by flow cytometry (Figure 5.8). In both groups, CCR4 expression was found to be significantly higher on the CLA+ T cells, confirming previous findings (Mean old: 70.37% vs 22.45%; mean young: 60.65%vs13.7% for CLA+ and CLA- cells respectively). No difference was found for CCR4 expression between the young and old groups (Mann Whitney CLA+ cells: p=0.15, CLA- cells p=0.2) (Figure 5.6). Unfortunately, it was not possible to stain cells for CCR10 as a reliable monoclonal antibody was not commercially available at the time of this study.

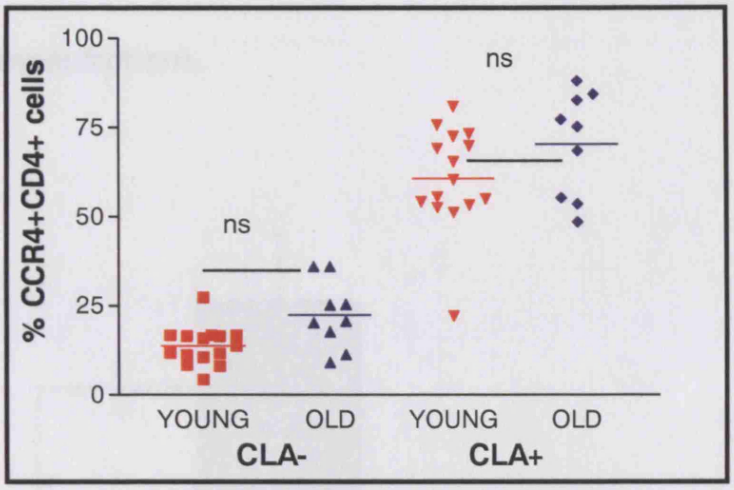


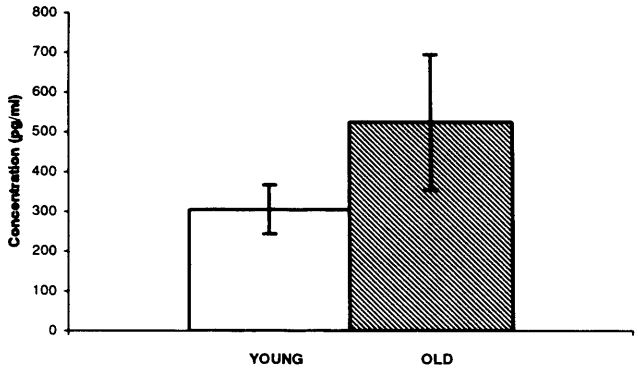
FIGURE 5.6. Expression of CCR4 on CLA+ and CLA- CD4+ T lymphocytes in the young and old.
PBMCs isolated from young and old individuals were stained with antibodies for CD3, CD4, CLA and CCR4 and analysed using flow cytometry (Figure 5.8). CD4+ lymphocytes were identified and the percentage of CCR4+ cells within the CD4+ lymphocyte population was calculated for CLA+ and CLA- populations. No significant difference was found for the percentage of CD11a+ cells within the CD4+CLA- (Mann Whitney p=0.2 or CD4+CLA+ cell (Mann Whitney p=0.15) populations for the old and young groups.

5.3.2. CCL17 and CCL27 concentrations

In order to determine whether levels of CCL17 and CCL27 expression within the skin differed in young and old subjects, skin suction blisters were raised over the site of Candin antigen injection at day 3. The levels of CCL17 and CCL27 within skin suction blister were then determined using a luminex technique. The luminex assays were performed by a collaborator (John Curnow) at Birmingham University. The day 3 time point was chosen for suction blister fluid analysis as this had previously been shown to be the time at which peak levels of the chemokines were present for the MT.

No difference was found for levels of CCL17 (Mann Whitney p=0.928) or CCL27 (Mann Whitney p=0.717) in the blister fluid from blisters induced over the site of the Candin skin test in young or old groups (Figure 5.7). Of note, no correlation was found between the clinical score measured in the skin at day 3 and the level of chemokine detected in either group (CCL17:Spearman r old=-0.31 p=0.356, Spearman r young=-0.48 p=0.2667; CCL27: Spearman r old= 0.36, p=0.27, Spearman r young=0.48, p=0.2667).

A. CCL27 concentrations.



B. CCL17 concentrations

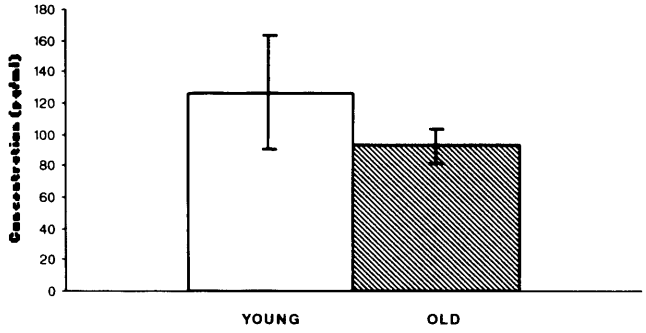
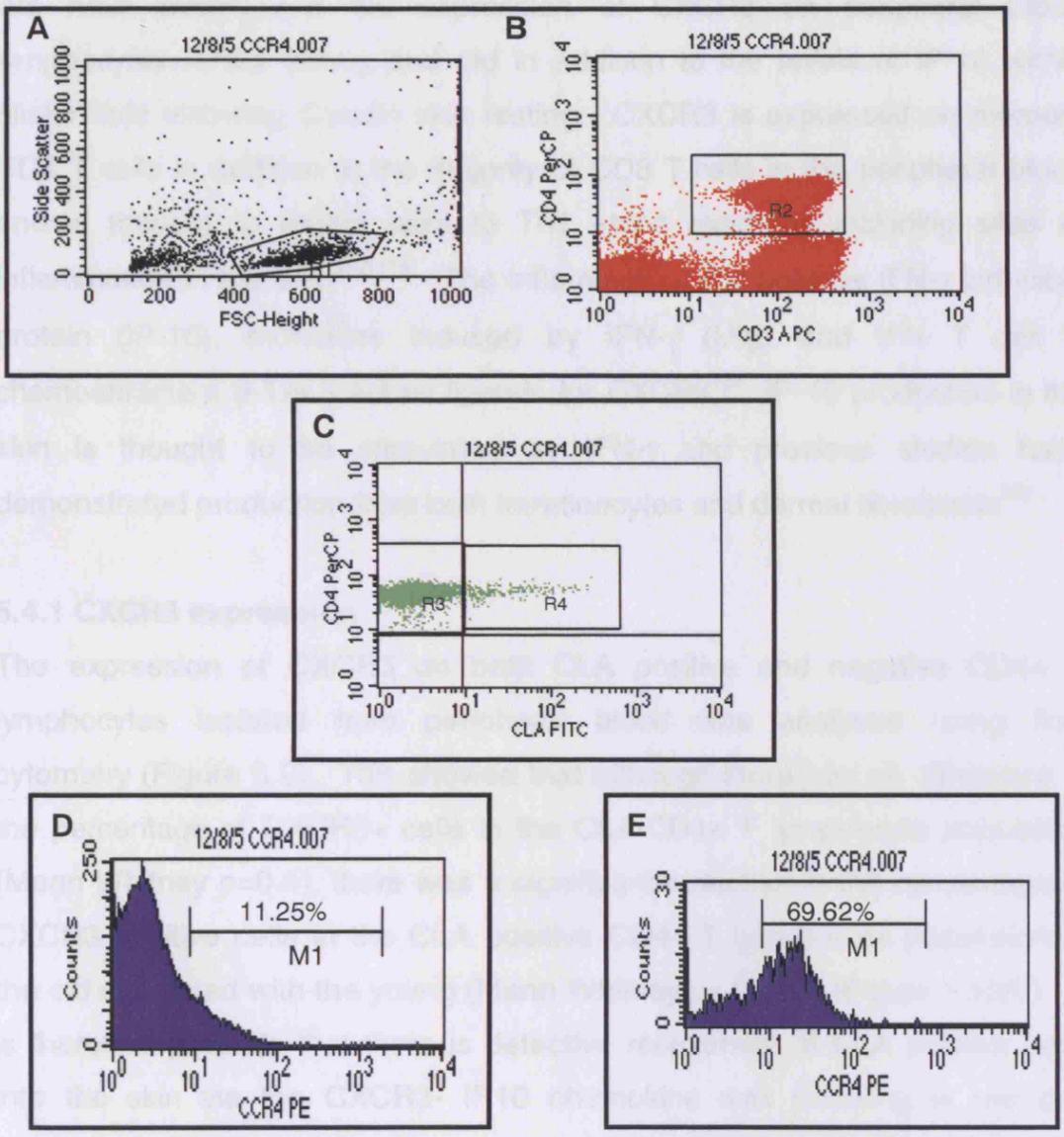


FIGURE 5.7. Concentrations of CCL27 and CCL17 in blister fluid.
Concentrations of the chemokines CCL27 (A) and CCL17 (B) were determined in blister fluid aspirated from blisters induced at Day 3 over the site of Candin injection in the young (n=7) and old (n=11). The concentrations of chemokines were calculated using a multiplex bead immunoassay (Luminex technique), performed by collaborator Dr. John Curnow at Birmingham University. No significant difference in the concentrations of either CCL27 (Mann Whitney p=0.717) or CCL17 (Mann Whitney p=0.929) were identified between the old and young. Error bars represent the SEM.



CLA- CD4+ LYMPHOCYTES (R3) CLA+CD4+LYMPHOCYTES (R4)

FIGURE 5.8. CCR4+ expression on CLA+ and CLA- CD4+ T lymphocytes PBMCs collected from young and old individuals were stained with anti- CD4 PercP, CD3 APC, CLA FITC and CCR4 PE. Live lymphocytes were identified and gated on the basis of the FSC/SSC profile in order to exclude cell debris **(A).** CD4+ cells were gated on the basis of CD3+and CD4+ staining on the lymphocyte gate **(B).** CLA+CD4+ cells (R3 gate) and CLA-CD4+ cells (R4 gate) were identified **(C).** Histograms were constructed based on gating for CLA-CD4+ cells (R3) **(D)** and CLA+CD4+ cells (R4) **(E).** The M1 gate was used to determine the percentage of CCR4+ cells for both cell populations (shown on histograms) and was set according to a negative isotype control for PE.

5.4 CXCR3 and IP-10 expression

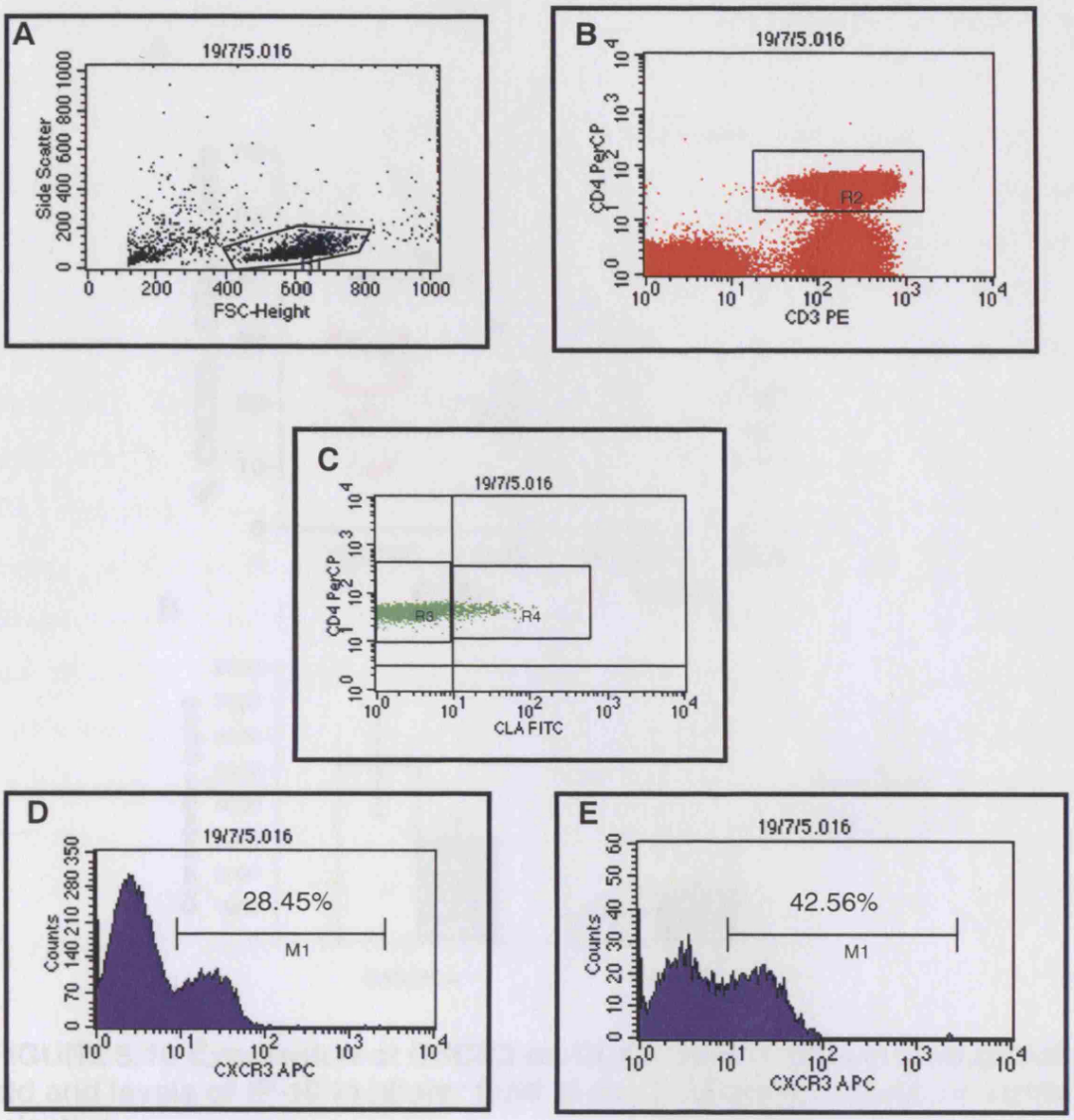
We next investigated the expression of CXCR3 on peripheral blood lymphocytes in the young and old in addition to the levels of IP-10 within blister fluid following Candin skin testing. CXCR3 is expressed on memory CD4 T cells in addition to the majority of CD8 T cells in the peripheral blood and is thought to attract cells to Th1 –type reactions including sites of inflammation in the skin[122;123]. The inflammatory chemokines IFN-γ inducible protein (IP-10), monokine induced by IFN-γ (Mig) and IFN T cell α chemoattractant (I-TAC) act as ligands for CXCR3[124]. IP-10 production in the skin is thought to be stimulated by IFN-γ and previous studies have demonstrated production from both keratinocytes and dermal fibroblasts[297].

5.4.1 CXCR3 expression

The expression of CXCR3 on both CLA positive and negative CD4+ T lymphocytes isolated from peripheral blood was analysed using flow cytometry (Figure 5.9). This showed that although there was no difference in the percentage of CXCR3+ cells in the CLA-CD4+ T lymphocyte population (Mann Whitney p=0.4), there was a significant reduction in the percentage of CXCR3 positive cells in the CLA positive CD4+ T lymphocyte population in the old compared with the young (Mann Whitney p= 0.023) (Figure 5.10A.) It is therefore possible that there is defective recruitment of CLA positive cells into the skin via the CXCR3- IP10 chemokine axis resulting in reduced immunity to Th1 type immune responses such as those seen for the MT and Candin skin test used in this study.

5.4.2 IP-10 concentrations

IP-10 levels were analysed in skin suction blister fluid from blisters raised over the Candin skin test and also after the injection of saline. No significant difference was found for the blister fluid levels of IP10 in both young and old after Candin skin testing (Mann Whitney p=0.34). (Figure 5.10B) Low levels of IP-10 only were found at sites of saline injection. No correlation was found between the clinical score at day 3 and the levels of IP-10 in either young (Spearman r=0.33, p=0.44) or old (Spearman r=0.57, p=0.07) groups.



CLA- CD4+ LYMPHOCYTES (R3) CLA+CD4+LYMPHOCYTES (R4)

FIGURE 5.9. CXCR3 expression on CLA+ and CLA- CD4+ T lymphocytes PBMCs collected from young and old individuals were stained with anti- CD4 PercP, CD3 PE, CLA FITC and CXCR3 APC. Live lymphocytes were identified and gated on the basis of the FSC/SSC profile in order to exclude cell debris **(A).** CD4+ cells were gated on on the basis of CD3+and CD4+ staining on the lymphocyte gate **(B).** CLA+CD4+ cells (R3 gate) and CLA-CD4+ cells (R4 gate) were identified **(C).** Histograms were constructed based on gating for CLA-CD4+ cells (R3) **(D)** and CLA+CD4+ cells (R4) **(E).** The M1 gate was used to determine the percentage of CXCR3+ cells for both cell populations (shown on histograms) and was set according to a negative isotype control for APC.

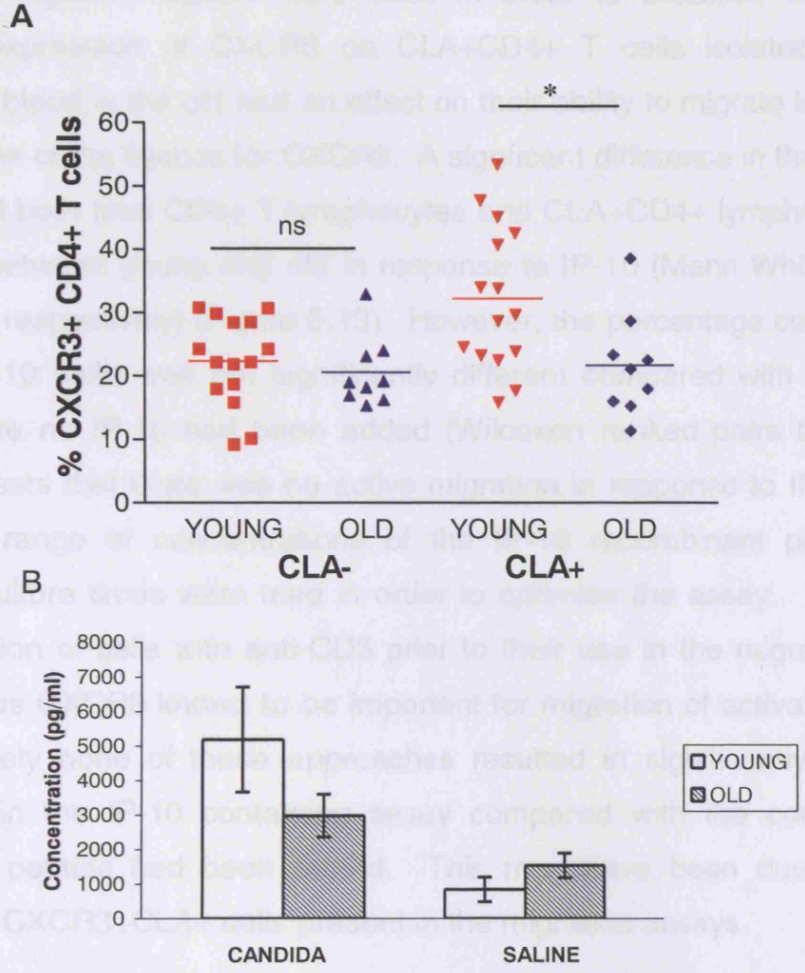


FIGURE 5.10 Expression of CXCR3 on CLA+ CD4+ T cells in young and old and levels of IP-10 in blister fluid at day 3 following Candin or saline injection.

A. PBMCs isolated from whole blood in young and old were stained with antibodies for CD3, CD4, CLA and CXCR3 and analysed using flow cytometry. Lymphocytes were selected for analysis on the basis of forward and side-scatter profiles. CD4+ lymphocytes were then identified and the percentage of CXCR3+ cells within the CD4+ lymphocyte population was calculated for CLA+ and CLA- populations (Figure 5.10). No significant difference was found for the percentage of CXCR3+ cells within the CD4+CLA- cell population (Mann Whitney p=0.023), however a reduction in the percentage of CXCR3+ cells was found in the CD4+CLA+ lymphocyte population in the old compared with the young (Mann Whitney p=0.4). B. IP-10 concentrations were measured in blister fluid from blisters raised at Day 3 following either Candin (n=6 young, n=11 old) or Saline injection (n=3 for both groups). Levels of IP-10 were determined using the BD^{TM} Cytometric Bead Array (CBA) Cell Signalling Flex Set System. No significant difference was found in the levels of IP-10 following injection of Candin (Mann Whitney p=0.34) or saline in the young and old groups. Error bars represent tthe SEM.

5.5 Transwell migration assays: migration of CLA+ CD4+ T lymphocytes

Transwell migration assays were used in order to establish whether the reduced expression of CXCR3 on CLA+CD4+ T cells isolated from the peripheral blood in the old had an effect on their ability to migrate in response to IP10, one of the ligands for CXCR3. A signficant difference in the migratory capacity of both total CD4+ T lymphocytes and CLA+CD4+ lymphocytes was detected between young and old in response to IP-10 (Mann Whitney P=0.4 and p=0.4 respectively) (Figure 5.13). However, the percentage cell migration for the IP-10 wells was not significantly different compared with the control wells where no IP-10 had been added (Wilcoxon ranked pairs test p>0.5). This suggests that there was no active migration in response to IP-10 in this assay. A range of concentrations of the IP-10 recombinant peptide and different culture times were tried in order to optimise the assay. In addition, pre-activation of cells with anti-CD3 prior to their use in the migration assay was tried as CXCR3 known to be important for migration of activated T cells. Unfortunately none of these approaches resulted in significantly increased migration in the IP-10 containing assay compared with the control assay where no peptide had been added. This may have been due the small number of CXCR3+CLA+ cells present in the migration assays.

The migration of total CD4+ and CLA+CD4+ lymphocyte populations in response to SDF was assessed. This assay was used as a positive control as the majority of T lymphocytes are known to express the CXCR4 receptor. Using flow cytometric analysis, the expression of CXCR4 on CLA+ CD4+ cells from the peripheral blood was compared in the young and old (Figure 5.11). No significant difference in CXCR4 expression on CLA+CD4+ T lymphocytes was found (Mann Whitney p=0.16)(figure 5.12A). However, a significant increase in the expression of CXCR4 on the CLA- population was found in the old compared with the young (Mann Whitney p=0.003), the reasons for which are unclear. Analysis of the levels of SDF in suction blister fluid taken from blisters at day 3 after Candin injection showed no significant difference in levels between young and old (Mann Whitney p=0.277)(Figure 5.12.B). Total CD4+ and CLA+CD4+ T lymphocytes were found to migrate in response to SDF in both young and old individuals with significantly higher numbers of

cells migrating compared with the control wells where no peptide had been added ($p>0.5$ for all SDF assays). No significant difference in the percentage of CLA+CD4+ or total CD4+ cells (Mann Whitney $p=1$ and $p=0.4$ respectively) migrating in response to SDF was found between the young and old (Figure 5.13 B+D), suggesting that the migratory capacity of CLA+CD4+ T lymphocytes in the old is not impaired *in vitro*.

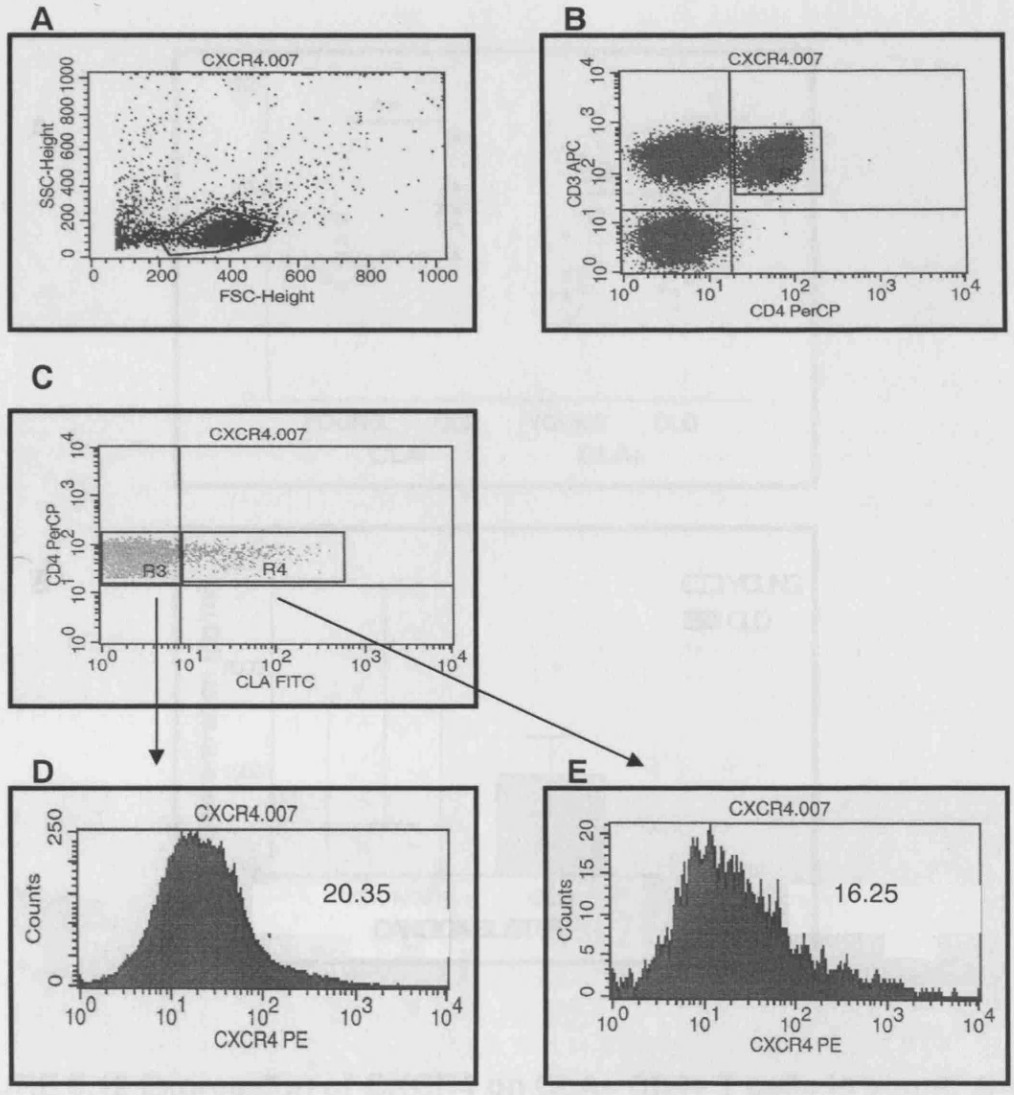


FIGURE 5.11. CXCR4 expression on CLA+ and CLA- CD4+ T lymphocytes

PBMCs collected from young and old individuals were stained with anti- CD4 PercP, CD3 APC, CLA FITC and CXCR4 PE. Live lymphocytes were identified and gated on the basis of the FSC/SSC profile in order to exclude cell debris (A). CD4+ cells were gated on on the basis of CD3+and CD4+ staining on the lymphocyte gate (B). CLA+CD4+ cells (R3 gate) and CLA-CD4+ cells (R4 gate) were identified (C). Histograms were constructed based on gating for CLA-CD4+ cells (R3) (D) and CLA+CD4+ cells (R4) (E) and the median fluorescent intensity (MFI) was calculated for both cell populations (values shown in this representative individual on histograms D and E)._

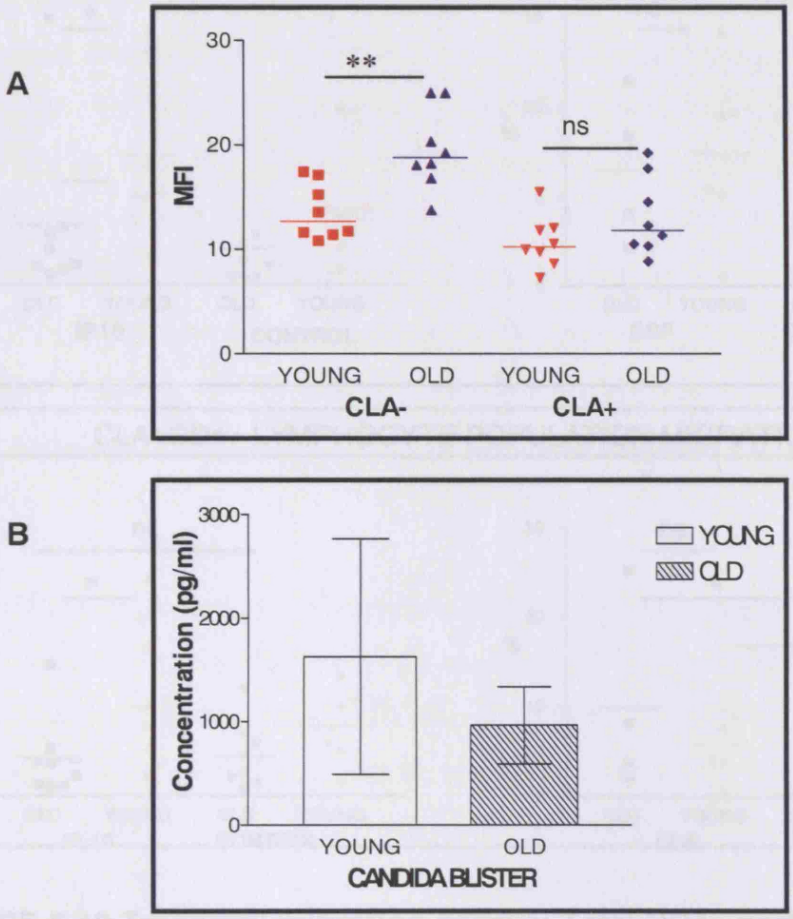


FIGURE 5.12 Expression of CXCR4 on CLA+ CD4+ T cells in young and old and levels of SDF in blister fluid at Day 3 following the Candin skin test.

A. PBMCs isolated from whole blood in young and old were stained with antibodies for CD3, CD4, CLA and CXCR4 and analysed using flow cytometry (Figure 5.11). CD4+ lymphocytes were identified and the percentage of CXCR4+ cells within the CD4+ lymphocyte population was calculated for CLA+ and CLA- populations. No significant difference was found for the percentage of CXCR4+ cells within the CD4+CLA+ cell population (Mann Whitney p=0.16), however a reduction in the percentage of CXCR4+ cells was found in the CD4+CLA- lymphocyte population in the young compared with the old (Mann Whitney p=0.003).

B. SDF concentrations were measured in blister fluid from blisters raised at Day 3 following Candin injection in young (n=7) and old (n=11) individuals. The concentrations of chemokines were calculated using a multiplex bead immunoassay (Luminex technique), performed by collaborator Dr. John Curnow at Birmingham University. No significant difference was found in the levels of SDF in blister fluid in the young and old groups (Mann Whitney p=0.277). Error bars represent the SEM

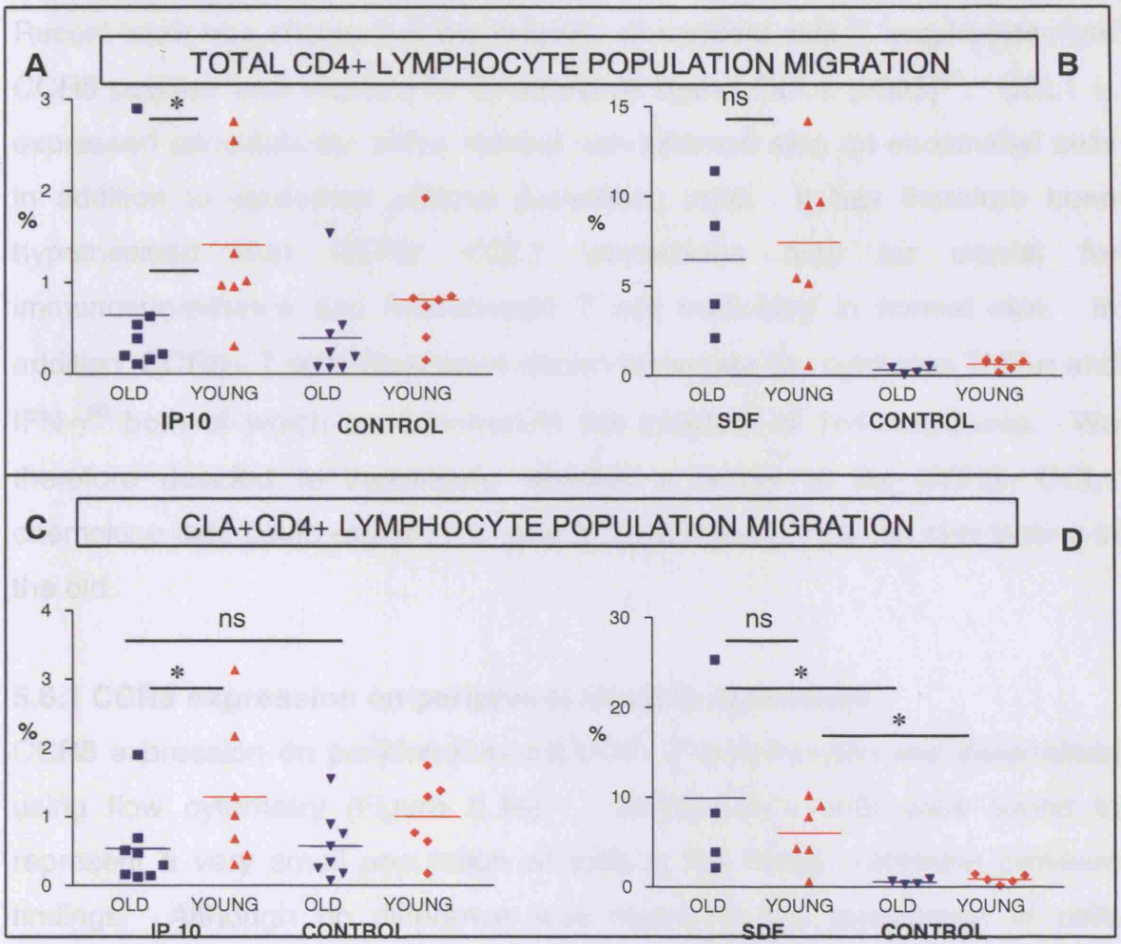


FIGURE 5.13 Transwell migration assays: Total CD4+ and CLA+CD4+ T cell migration in response to IP 10 and SDF.

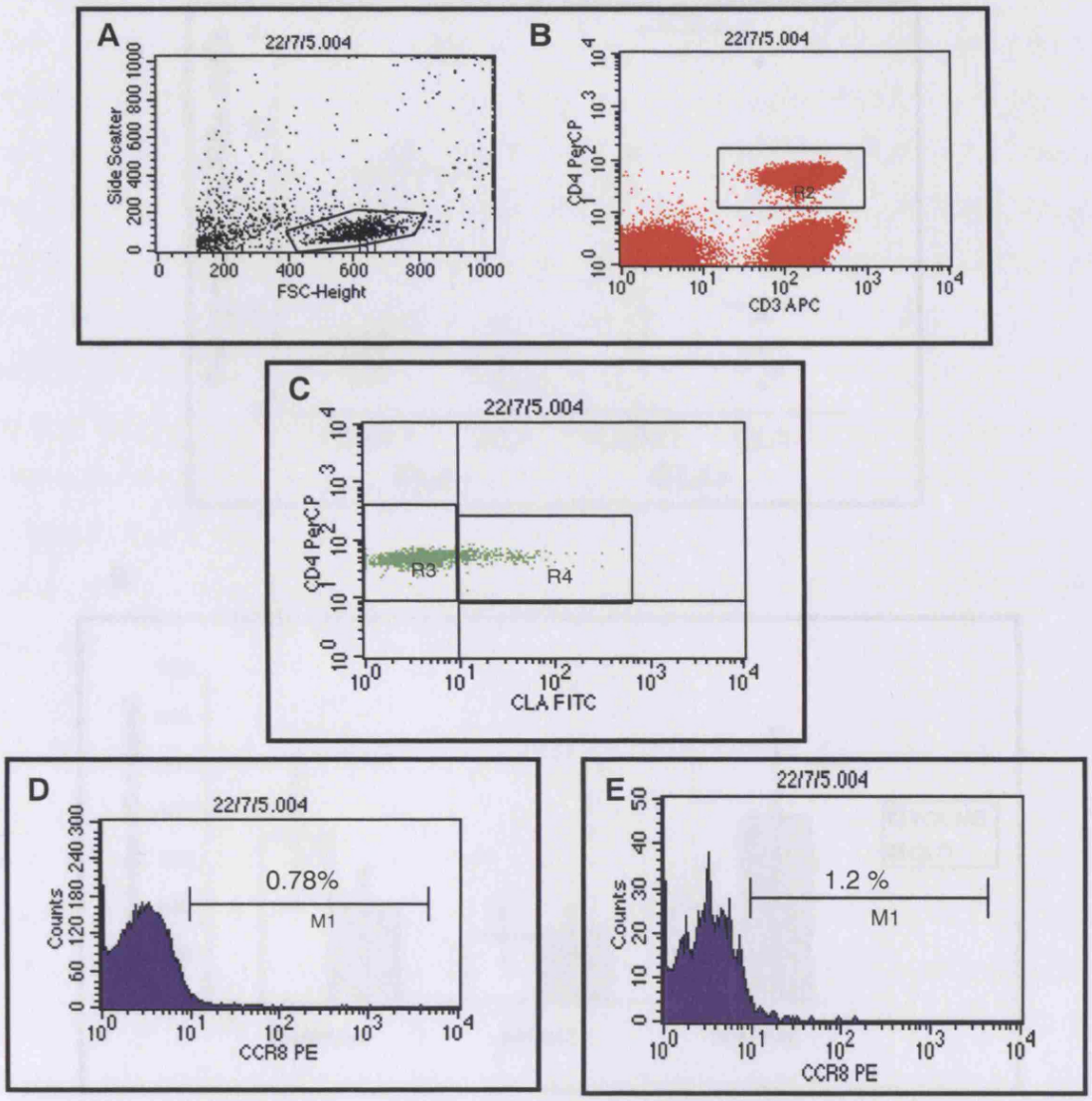
In vitro cell migration assays were used to quantify the ability of total CD4+ and CD4+CLA+ T lymphocytes to migrate in response to IP-10 (I-309) and SDF recombinant peptides. SDF was used as a positive control as most cells (>80%) express its ligand CXCR4. As a negative control culture medium alone was used. PBMCs were isolated from young and old individuals. Chambers containing 3μm size pore Transwell inserts (top chambers) were placed into the wells of 26 well flat-bottomed plates (bottom chambers) containing cell culture medium and recombinant peptide. PBMCs suspended in culture medium were then added to the top chambers. Triplicate wells were used for each individual's PBMCs and each recombinant peptide. The percentage of cells migrating through the Transwell insert from the top chamber into the bottom chamber after 3 hours of incubation was calculated. Cells were retrieved from the bottom and top chambers and stained with anti-CLA, CD3 and CD4 antibodies in a Trucount tube, allowing for calculation of cell numbers by flow cytometry. There was a reduction in the ability of total CD4 (A+B) and CLA+ CD4+ (C+D) T lymphocytes to migrate in response to the IP-10 (A+C) in old compared with young individuals (Mann Whitney p=0.04 for both subsets). No difference in the ability of cells to migrate in response to SDF (B+D) peptides was found (Mann Whitney p=1 (CLA+CD4+) and p=0.76 (CD4+). For the IP-10 peptide similar numbers of cells migrated in the control wells (with no peptide) however a significant reduction in cell migration was seen in the control wells compared with SDF.

5.6 CCR8 and CCL1 (I-309) expression

Recent work has shown that the majority of resident skin T lymphocytes are CCR8 positive and respond to its selective ligand CCL1 (I-309)[66]. CCL1 is expressed constitutively within normal non-inflamed skin on endothelial cells in addition to epidermal antigen presenting cells. It has therefore been hypothesised that CCR8/ CCL1 interactions may be crucial for immunosurveillance and homeostatic T cell trafficking in normal skin. In addition, CCR8+ T cells have been shown to secrete the cytokines TNF-α and IFN-γ[66] both of which are involved in the initiation of Th1 responses. We therefore decided to investigate whether a defect in the CCR8- CCL1 chemokine axis could explain the reduced response to Candin skin testing in the old.

5.6.1 CCR8 expression on peripheral blood lymphocytes

CCR8 expression on peripheral blood CD4+ T lymphocytes was determined using flow cytometry (Figure 5.14). CCR8+CD4+ cells were found to represent a very small population of cells in the blood confirming previous findings. Although no difference was found for the percentage of cells expressing CCR8 in the total CD4+ T lymphocyte population in both young (mean: 0.83%) and old (mean:0.68%) individuals (Mann Whitney p=0.12), a statistically significant reduction in the percentage of cells with the CCR8+CD4+CLA+phenotype was found in the old (mean: 0.61%) compared with the young (mean: 1.65%) groups (Mann Whitney p=0.0007). (Figure 5.15A). However, due to the very low percentages of positive cells the accuracy of this finding is uncertain.



CLA- CD4+ LYMPHOCYTES (R3) CLA+CD4+LYMPHOCYTES (R4)

FIGURE 5.14 CCR8+ expression on CLA+ and CLA- CD4+ T lymphocytes PBMCs collected from young and old individuals were stained with anti- CD4 PercP, CD3 APC, CLA FITC and CCR8 PE. Live lymphocytes were identified and gated on the basis of the FSC/SSC profile in order to exclude cell debris **(A).** CD4+ cells were gated on on the basis of CD3+and CD4+ staining on the lymphocyte gate **(B).** CLA+CD4+ cells (R3 gate) and CLA-CD4+ cells (R4 gate) were identified **(C).** Histograms were constructed based on gating for CLA-CD4+ cells (R3) **(D)** and CLA+CD4+ cells (R4) **(E).** The M1 gate was used to determine the percentage of CCR8+ cells for both cell populations (shown for this representative individual) and was set according to a negative isotype control for PE.

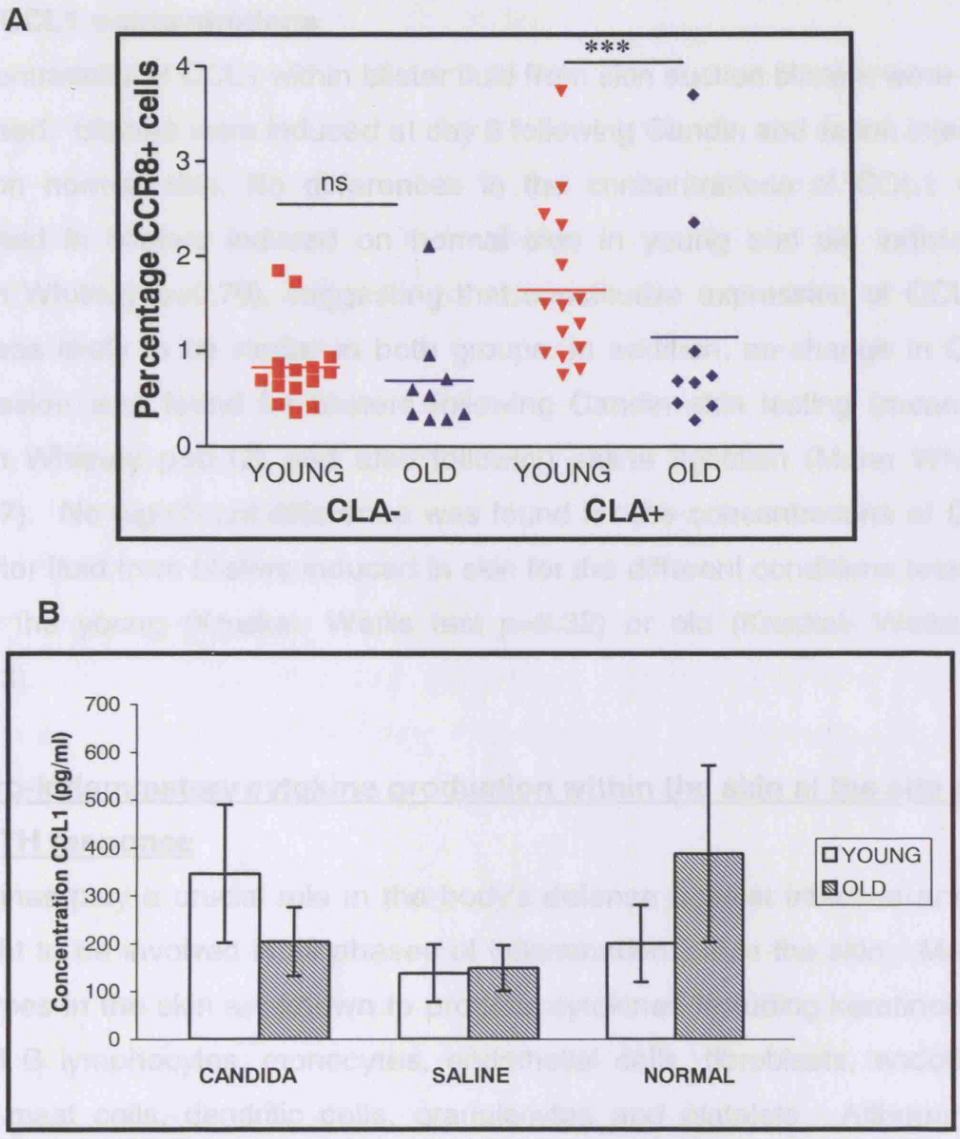


FIGURE 5.15 Expression of CCR8 on CLA+ CD4+ T cells in young and old and levels of CCL1 in blister fluid.

A. PBMCs isolated from whole blood in young and old were stained with antibodies for CD3, CD4, CLA and CCR8 and analysed using flow cytometry (Figure 5.14). CD4+ lymphocytes were identified and the percentage of CCR8+ cells within the CD4+ lymphocyte population was calculated for CLA+ and CLA- populations. No significant difference was found for the percentage of CCR8+ cells within the CD4+CLA- cell population, however a reduction in the percentage of CCR8+ cells was found in the CD4+CLA+ lymphocyte population in the old compared with the young (Mann Whitney p=0.0007).

B. CCL1 concentrations were measured in blister fluid from blisters raised on normal skin or at Day 3 following either Candin (n=6 young, n=11 old) or saline injection (n=3 in both groups) in young and old individuals. Levels of CCL1 were determined using the BD™ Cytometric Bead Array (CBA) Cell Signalling Flex Set System. No significant difference was found in the levels of CCL1 in blister fluid from normal skin or following injection of Candin or saline in the young and old groups. Error bars represent the SEM.

5.6.2 CCL1 concentrations

Concentrations of CCL1 within blister fluid from skin suction blisters were then analysed. Blisters were induced at day 3 following Candin and saline injection and on normal skin. No differences in the concentrations of CCL1 were identified in blisters induced on normal skin in young and old individuals (Mann Whitney p=0.70), suggesting that constitutive expression of CCL1 in skin was likely to be similar in both groups. In addition, no change in CCL1 expression was found for blisters following Candin skin testing (mean old: (Mann Whitney p=0.12) and also following saline injection (Mann Whitney p=0.87). No significant difference was found for the concentrations of CCL1 in blister fluid from blisters induced in skin for the different conditions tested in either the young (Kruskal- Wallis test p=0.32) or old (Kruskal- Wallis test p=0.43).

5.7 Pro-inflammatory cytokine production within the skin at the site of the DTH response

Cytokines play a crucial role in the body's defense against infection and are thought to be involved in all phases of inflammation within the skin. Multiple cell types in the skin are known to produce cytokines including keratinocytes, T and B lymphocytes, monocytes, endothelial cells, fibroblasts, endothelial cells, mast cells, dendritic cells, granulocytes and platelets. Although the isolation of different cytokines *in vitro* has enabled the study of individual cytokine function, *in vivo*, a cocktail of cytokines will exert a variety of both synergistic and antagonistic effects on different cells within the local microenvironment in order to orchestrate an effective immune response. The cytokines TNF, IL-6 and interferon gamma have been shown to have pro-inflammatory effects on the skin and are likely to be important in the initial induction of the response to cutaneous infection, known as the acute phase response. We therefore assessed levels of these cytokines in the skin following the injection of Candin.

5.7.1 Tumor necrosis factor (TNF) levels

TNF is secreted by a variety of cells in the skin including monocytes and macrophages, T cells, keratinocytes and endothelial cells and is thought to have multiple pro-inflammatory activities. TNF has been found to increase macrophage and neutrophil chemotaxis, phagocytosis, cytotoxicity and respiratory burst activity. TNF-α has important effects on blood vessels, in particular venules, to increase blood flow and to increase vascular permeability to fluid, proteins and cells. Local release thus results in erythema due to vasodilatation in addition to swelling due to influx of fluid in to the infected tissue. It also promotes the adherence of T lymphocytes to the endothelium by increasing the expression of E-selectin, ICAM-1 and VCAM on the endothelial cell surface. TNF-α has also been shown to be important for the maturation of dendritic cells in humans and their migration from the epidermis in to the dermis and may therefore have an important early role in the initiation of immune responses.

TNF levels were measured in skin suction blisters raised over the site of Candin and saline injection at Day 3 (Figure 5.16). A significant reduction in the level of TNF was noted in the old group compared with the young in the skin after Candin injection (Mann Whitney p=0.013). Low levels of TNF were found in blister fluid after saline injection indicating that TNF production was induced by the injection of antigen rather than by trauma alone in the young. In the young and old, no correlation between TNF production and clinical score was found (Young: Spearman r=0, p=1.03, Old: Spearman r=0.43, p=0.18).

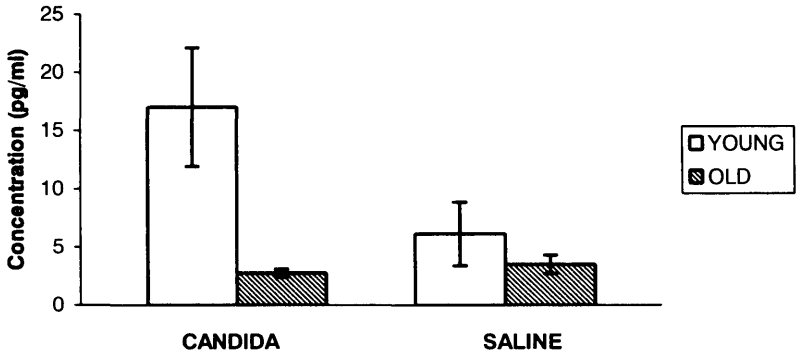


FIGURE 5.16 Concentrations of TNF in blister fluid from blisters induced at day 3 following Candin and saline injection.

Concentrations of TNF were determined in blister fluid aspirated from blisters induced at Day 3 over the site of Candin injection (n=6 young, n=11 old) and following saline injection (n=3 both groups) in the young and old. The concentrations of TNF were calculated using the BD™ Cytometric Bead Array (CBA) Cell Signalling Flex Set System. A significant reduction in the concentration of TNF was found in the old following Candin injection compared with the young (Mann Whitney p=0.013). Error bars show the SEM.

5.7.2 Interferon gamma (IFN-γ) levels.

IFN-γ is produced predominantly by activated T lymphocytes. It has been shown to have several pro-inflammatory effects including the up regulation of MHC Class II expression on monocytes[103] and keratinocytes[298] in addition to the activation of monocytes with increased secretion of TNF-α[104].

IFN-γ concentrations were measured in skin suction blisters as for TNF (Figure 5.17). Although there was an increase in the mean concentration of IFN-γ production in the blister fluid raised over the site of Candin skin testing at day 3 in the young compared with the old, this failed to reach statistical significance (Mann Whitney p=0.118). This is possibly reflective of the small sample size collected. Low levels of IFN-γ were detected in blisters following saline injection, indicating that trauma alone is not sufficient to induce significant production of IFN-γ within the skin. IFN-γ levels were independent

of clinical score at day 3 in both young (Spearman r=0.70, p=0.09) and old individuals (Spearman r=0.50, p=0.11).

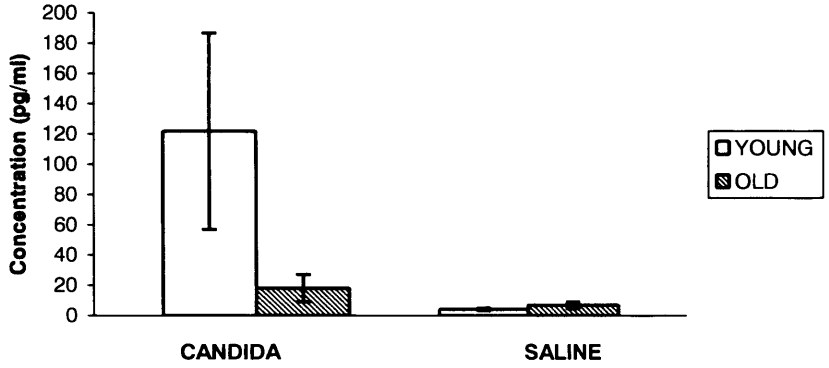


FIGURE 5.17 Concentrations of IFN-γ in blister fluid from blisters induced at day 3 following Candin and saline injection.

Concentrations of IFN-γ were determined in blister fluid aspirated from blisters induced at Day 3 over the site of Candin injection (n=6 young and n=11 old) and following saline injection in the young and old (n=3 for both groups). The concentrations of IFN-γ were calculated using the BD^{TM} Cytometric Bead Array (CBA) Cell Signalling Flex Set System. A reduction in the concentration of IFN-γ was found in the old following Candin injection compared with the young but this was not signficant (Mann Whitney p=0.118). Error bars show the standard error of the mean. In the old there was no significant difference between the blisters following Candin or saline injection (Wilcoxon ranked pairs test p>0.5).

5.7.3 IL-6 levels

IL-6 concentrations were measured in skin suction blisters as for TNF and IFN-γ. Monocytes are the main producers of IL-6 in the skin, although other cell types including keratinocytes, endothelial cells and fibroblasts have also been reported to secrete IL-6[299]. In T cells IL-6 mediates activation, growth and differentiation[105;106] and is considered to be an important early pro-inflammatory cytokine involved in amplifying the immune response.

No significant difference was found for blisters in young and old over site of Candin injection (Mann Whitney p=0.06) (Figure 5.18). Low concentrations of IL-6 were found in young and old blisters induced at day 3 after saline injection.

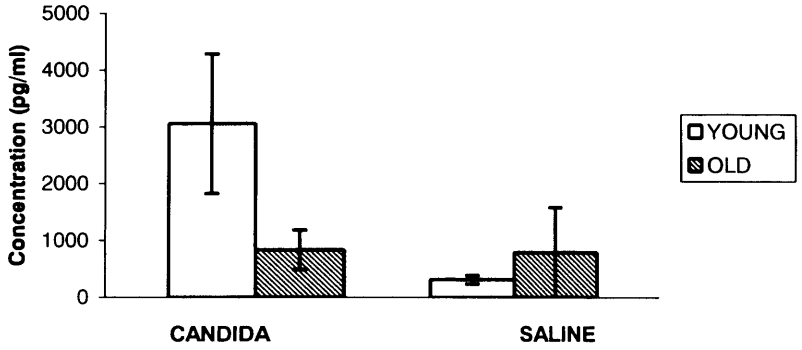


FIGURE 5.18 Concentrations of IL-6 in blister fluid from blisters induced at day 3 following Candin and saline injection.

Concentrations of IL-6 were determined in blister fluid aspirated from blisters induced at Day 3 over the site of Candin injection (n=6 young and n=11 old) and following saline injection (n=3 for both groups) in the young and old. The concentrations of IL-6 were calculated using the BD™ Cytometric Bead Array (CBA) Cell Signalling Flex Set System. No significant difference in the concentration of IL-6 was found in the old following Candin injection compared with the young (Mann Whitney p=0.06). Error bars show the standard error of the mean. In the old there was no significant difference between the blisters following Candin or saline injection (Wilcoxon ranked pairs test p>0.5).

5.8 Immunosuppressive cytokines: IL-10 levels

IL-10 levels in blister fluid were measured in both young and old. In addition to pro-inflammatory cytokines, certain cytokines such as IL10 are able to exert potent anti-inflammatory effects. IL-10 has been shown to inhibit the release of several cytokines including TNF-α production from monocytes. It is produced by both monocytes and lymphocytes including regulatory T cells.

No difference was found for concentrations of IL-10 in blister fluid from blisters raised at day 3 over the site of Candin injection in both young and old blisters (Mann Whitney:p=0.88) (Figure 5.19). In addition, similar levels of IL-10 were found in blisters raised at day 3 after saline injection in young and old (Mean young:12.35pg/ml, mean old:9.27pg/ml; Wilcoxon ranked pairs test young p=0.5, old p=0.25), suggesting that IL-10 production is not significantly up regulated during Candida antigen induced responses in the skin.

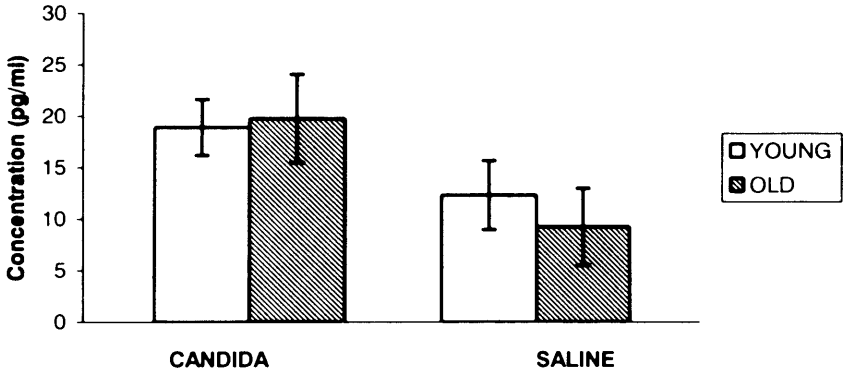


FIGURE 5.19 Concentrations of IL-10 in blister fluid from biisters induced at day 3 following Candin and saline injection.
Concentrations of IL-10 were determined in blister fluid aspirated from blisters induced at Day 3 over the site of Candin injection (n=6 young, n=11 old) and following saline injection (n=3 both groups) in the young and old. The concentrations of IL-10 were calculated using the BD™ Cytometric Bead Array (CBA) Cell Signalling Flex Set System. No significant difference in the concentration of IL-10 was found in the old following Candin injection compared with the young (Mann Whitney p=0.88). There was no significant difference between the blister fluids following Candin or saline injection (Wilcoxon ranked pairs test p=0.5 young, p=0.25 old). Error bars show the standard error of the mean.

5.9 Chemokines involved in amplification of the immune response: RANTES, MCP-1, MIP- 1α, IL-8.

Following the acute initiation of the immune response in the skin, a variety of cell types secrete a range of chemokines that are able to amplify the response by cellular activation and increased cellular recruitment to the site of inflammation in the skin as shown in Table 1.2 (Introduction).

A statistically significant reduction in the concentrations of MIP-1α (Mann Whitney p=0.008), MCP-1 (Mann Whitney p=0.0057), RANTES(Mann Whitney p=0.0182), and IL-8 levels (Mann Whitney:p=0.0056) in suction blister fluid raised at day 3 after Candin injection was observed in the old (Figure 5.20). High levels of IL-8 were measured in blister fluid from the young group after saline injection with no significant difference between levels in candida or saline blisters (Wilcoxon ranked pairs test p=0.5). As with TNF-α levels, the clinical score in either the old or young groups did not correspond with levels of chemokines measured. Spearman r values for old: RANTES (r=0.56, p=0.08), MCP-1 (r=0.06, p=0.86), MIP-1α (r=0.394, p=0.22), IL-8 and young: RANTES (r=0.41, p=0.35), MCP-1 (r=0.30, p=0.49), MIP-1α (r=0.59, p=0.17).

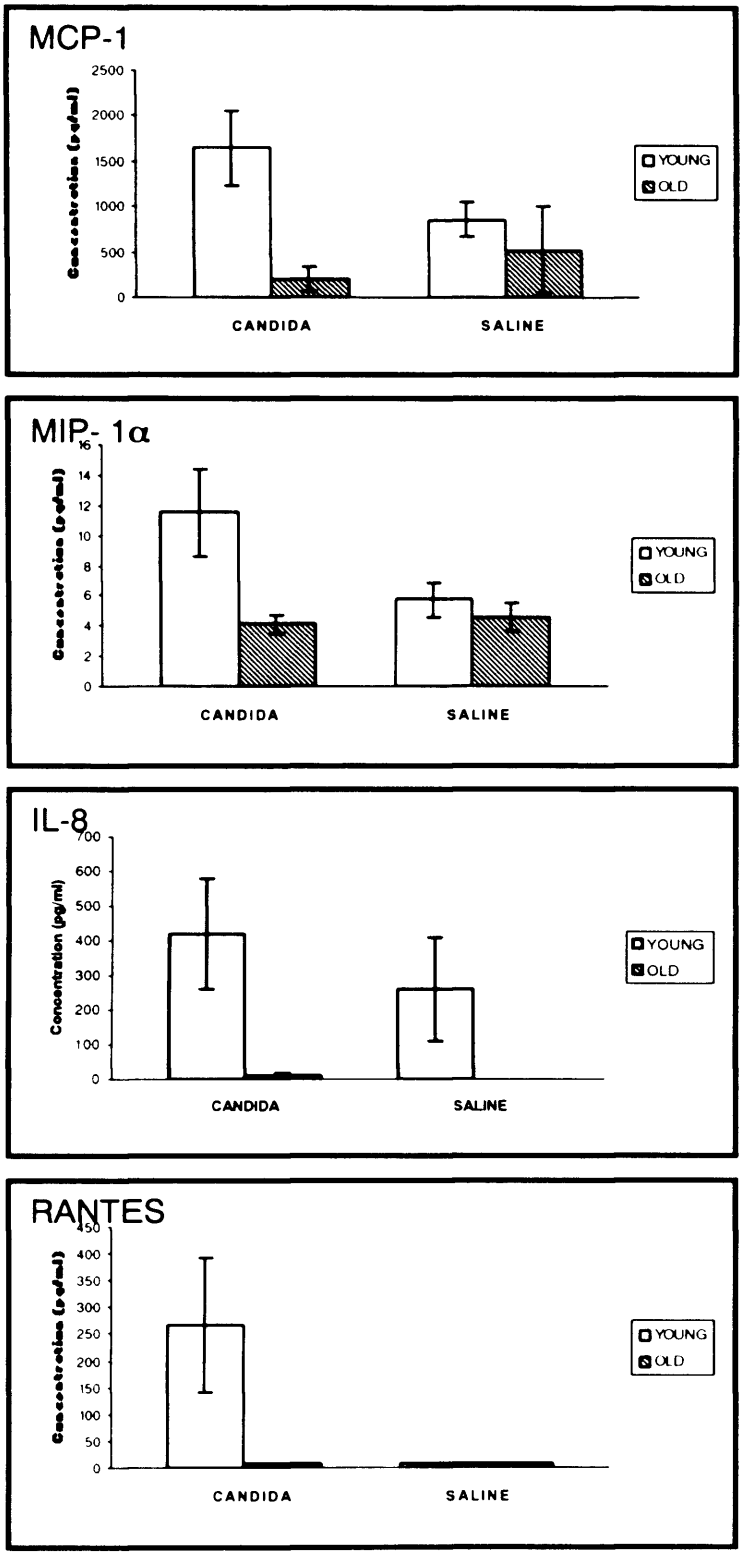


FIGURE 5.20 Concentrations of MCP-1, MIP-1α, RANTES, IL-8 in blister fluid from blisters induced at day 3 following Candin and saline injection.

Concentrations of chemokines were determined in blister fluid aspirated from blisters induced at Day 3 over the site of Candin injection (n=6 young, n=11 old) and following saline injection (n=3 in both groups) in the young and old. The concentrations of MCP-1, MIP-1α, RANTES and IL-8 were calculated using the BD™ Cytometric Bead Array (CBA) Cell Signalling Flex Set System. A significant reduction in the concentration of all chemokines/ cytokines was found in the old following Candin injection compared with the young (Mann Whitney $p<0.5$) for all measured concentrations. Error bars show the SEM.

5.10 Discussion

In this chapter we have investigated potential mechanisms for the apparent reduction in the number of lymphocytes present at the site of immune responses in the old. This could be representative of either reduced recruitment of cells to the site or reduced activation of cells recruited to the skin due to an altered cutaneous microenvironment. The production of chemokines and cytokines at the site of the immune response is crucial for coordination of immune responses, in particular for the initial recruitment of effector memory T cells, their activation in the skin and then down-regulation in order to enable resolution. Multiple cell types within the skin produce an array of chemokines and cytokines many of which have pleiotropic effects creating a complex milieu. Many cytokines and chemokines interact with each other and so it is difficult to assertain the precise role of individual factors when studying them in isolation *in vitro*.

We initially investigated whether circulating memory T cells from the peripheral blood had any detectable changes in skin homing phenotype. Circulating memory T cells can be divided in to tissue-specific subsets based on their expression of adhesion molecules and chemokine receptors. Effector memory T cells that are specialised for entry in to the skin are characterised by the expression of cutaneous lymphocyte antigen (CLA), the ligand for E-selectin. We have demonstrated that there is no change in the percentage of CLA expression both on the total lymphocyte population and also on the memory CD45RO+CD4+ T cell population in the young and old. There is evidence to suggest that CLA positive cells are a population of cells that do not undergo immunosenescence in that although a decrease in CD45RA and CD28 expression has been found for CD3+ CLA- cells, there was no change in expression of these markers in the CLA+ T cell population. Furthermore, telomere length did not shorten in the CLA+ T cell population but shortened in the CLA- T cell population[300].

We have also shown that there is no difference in the percentage of CLA+ T cells that produce IFN-γ in response to overnight stimulation with antigen *in vitro* in the young and old, suggesting that function of the CLA+CD4+ T cells is

not significantly impaired in the old. It is of interest that differences in CLA expression were seen for memory T cells of different antigenic specificities were seen in both the young and old. It is thought that naïve lymphocytes acquire tissue-specific tropism during the primary immune response. This is thought to occur within secondary lymphoid tissue associated with a particular tissue in which exposure to antigen has occurred[12]. This is supported by studies that have demonstrated that circulating T lymphocytes specific for cutaneous allergens have high levels of CLA expression[301].

All of the young volunteers in this study had received BCG vaccine, administered percutaneously, whereas none of the old individuals had been vaccinated. The old individuals in this study were likely to have developed immunity to the PPD antigen due to the exposure of both respiratory and gastrointestinal tract mucosa to environmental mycobacteria. It could therefore be predicted that there would be increased CLA expression on PPD-specific T lymphocytes in the young group due to administration of the antigen through the skin during vaccination. However, no difference was found in CLA expression on PPD specific CD4+Tlymphocytes in the young and old groups. Increased CLA expression was found for candida specific CD4+ T lymphocytes in both the young and old. This is possibly due to the repeated cutaneous exposure and/or colonisation of the skin with candida, allowing for maintenance of skin-specific homing memory T cell population for this antigen.

In addition to CLA, the chemokine receptors CCR4 and CCR10 have been proposed as critical mediators of skin specific T lymphocyte homing in humans and mice. We did not identify any differences in the expression of CCR4 expression on CLA+ T lymphocytes in the young and old. In addition, the expression of CCL17 and CCL27, the ligands for CCR4 and CCR10 respectively was also the same in the young and old.

We were unable to assess for CCR10 expression on peripheral blood lymphocytes due to the lack of a commercially available monoclonal antibody. It is unlikely however that a difference in the expression of CCR10 in the old is

responsible for the observed reduced immune response. CCR10 is only expressed on around 28% of CLA+ memory T lymphocytes and virtually all CCR10+ CD4+ T lymphocytes also express CCR4[117]. Whereas CCL17 (the ligand for CCR4) expression is found predominantly on dermal venules, CCL27 (the ligand for CCR10) is produced mainly by keratinocytes[87]. Thus while the CCR4-CCL17 axis is likely to be critical in our model, where infiltration of cells is predominantly dermal, the role of CCR10- CCL27 chemokine axis is likely to be redundant in that very few cells are seen to infiltrate the epidermis where the majority of CCL27 is expressed.

We did not identify any change in the expression of CD11a in the total lymphocyte population and also CD4+CLA+ T lymphocytes in young and old individuals. CD11a is a subunit of LFA-1, essential for the adhesion of T lymphocytes to the endothelium via binding to ICAM-1- expression of which is up regulated in inflamed skin. Both mouse[302] and human studies[303] have shown increase in LFA-1 expression on memory T cells with ageing and we would therefore have expected an increase in LFA-1 expression on T lymphocytes within the CLA positive (memory) T lymphocyte population. Differences in this study may reflect the fact that we were studying a healthy aging group of individuals without significant co-morbidity. However, in elderly humans there is also evidence for diminished cell-cell binding due to altered activation of LFA-1[304]. It is therefore possible that in spite of normal levels of LFA-1 in our old group there may be loss of function.

Although we have excluded changes in the expression of specific skin-homing receptor expression on peripheral blood CD4+ memory T lymphocytes as a reason for reduced lymphocyte numbers in the old, we have identified a reduction in the percentage of CLA+ T cells expressing CXCR3. Although not tissue-specific, CXCR3 is thought to be an important skin homing receptor[305], supported by the observations that a significant subset of CXCR3 positive CD4+ T lymphocytes also express the skin-specific receptor CCR4[123] and that skin derived cells (keratinocytes and monocytes) are able to produce the ligands for CXCR3[297]. Thus, a reduction in the expression of CXCR3 on

CLA+ T lymphocytes may explain the reduction in lymphocyte numbers at the site of antigenic challenge in the old.

In contrast to our findings in humans, murine studies using ageing mice have identified increase in CXCR3 expression (in addition to multiple other chemokine receptors) on peripheral blood CD4+ T cells associated with increased migratory capacity to migrate in response to CXCR3 ligands[306]. We did not identify any changes in the migratory capacity of CXCR3+ lymphocytes from old individuals in this study although this may reflect the limitations of the *in vitro* assay used. CXCR3 expression in the CLA+ T lymphocyte subset has not been previously investigated in humans, however increased CXCR3 expression on naïve subset of cells with ageing has been demonstrated and the authors propose that this may result in the inappropriate migration of naïve cells to unorthodox locations[307]. Previous studies in mice have shown increased production of the ligands for CXCR3 with ageing[308;309]. In this study the levels of IP-10 measured in young and old blister fluid were similar however we did not measure the concentrations of the other ligands for CXCR3 (MIG and I-TAC).

CXCR3 also thought to be important for cellular retention at sites of chronic inflammation such as rheumatoid arthritis[310] and chronic liver disease[126] with up regulation of the receptor on T lymphocytes at sites of chronic inflammation. The role of CXCR3 in cellular retention during an acute inflammatory response is unknown, however a reduction in CXCR3 expression in the old may indicate there is impaired T cell retention at sites of inflammation in the old. CXCR3 is thought to be up regulated on activated T cells[311]. The T lymphocytes derived from PBMCs in our study were not activated and so we cannot conclude if any differences are present in receptor expression at the sites of inflammation where T cells are likely to be activated.

In this study we also identified a possible reduction in the percentage of CCR8+CLA+ T lymphocytes in the peripheral blood, however this population was extremely small in both young and old individuals (less than 5% of T cells) making the accurate analysis of this subset of cells difficult. It has been

demonstrated that the majority of T cells in human skin express the CCR8 receptor and respond to CCL1 and it has been proposed that the CCL1-CCR8 chemokine axis provides a mechanism for homeostatic T cell traffic in the skin[66]. Similar levels of CCL1 were found in blisters induced over normal skin in the young and old, indicating that the old do not have deficient CCL1 production. No increases in the blister fluid concentrations of CCL1 were seen at Day 3 and 7 following Candin injection, suggesting that CCL1 has a role in homeostasis rather than in acute inflammation. We did not investigate the expression of CCR8 on lymphocytes resident in normal skin although we have demonstrated that there is no difference in the number of resident CD4+ and CD8+ T cells in young and old skin (Chapter 4).

We next went on to investigate whether any detectable changes present in cytokines produced as part of the acute phase response could be responsible for the reduced clinical and cellular response to antigen injection in the old. Variable levels of cytokine and chemokine production were seen in the young volunteers with poor correlation between clinical response and production of chemokine. The reasons for this are unclear. This may have been due to the small number of individuals tested. During acute inflammation, inflammatory mediators are not only produced but also consumed during the response and it is possible that individual variation in the balance between consumption and production of cytokine may have resulted in the observed variety of concentrations measured. Certain cytokines and chemokines are known to be labile and sensitive to freeze/ thaw cycles and this may have resulted in inconsistent deterioration in the samples. All of our blister fluid samples were frozen at -80°C immediately after they had been spun down in order to pellet the blister cells and the samples were only frozen and defrosted on one occasion. However, there was some variation in the time taken following aspiration of the blister fluid from the volunteer and freezing the sample that may have resulted in variations between samples. All samples were analysed for chemokine/cytokine levels in one run in order to minimise variation between runs. Certain chemokines were present in high concentrations after the injection of saline in to the skin. The induction of blisters in addition to the

introduction of a needle in to the skin is likely to be traumatic and may induce the non-specific release of chemokines.

A reduction in the mean levels of IFN-γ was observed. The injection of IFN-γ in to the skin of young humans has been shown to increase ICAM –1 expression on endothelial cells and keratinocytes with a corresponding infiltration of lymphocytes in to the skin[312]. In addition IFN-γ is thought to play an important role in the activation of monocytes/ macrophages with increased TNF-α production, expression of MHC class II molecules and enhanced monocyte antigen presenting function. In mice, reduced transcription of MHC Class II on macrophages in response to IFN-γ with ageing has been demonstrated[313]. The effects of ageing in humans on the response to IFN-γ injection have also been investigated in a previous study. This demonstrated a reduced accumulation of monocytes in addition to reduced HLA-DR expression on keratinocytes[314]. Thus, both a reduction in the production of IFN-γ in old skin in addition to reduced responsiveness to IFN-γ may result in the observed reduction in lymphocyte numbers and immune responses seen in our old individuals.

IFN-γ is predominantly made by memory Th1 effector T cells present in the skin at the site of antigen injection following antigen presentation. Our data indicates that this may be true in our model in that very little IFN-γ was found in blister fluid following the injection of saline, thus indicating that IFN-γ production is dependent on the injection of an antigen rather than due to trauma alone.

A significant reduction in the levels of TNF-α was found in old skin following Candin injection compared with the young. In addition to IFN-γ, TNF-α production is also thought to be important in the initiation of immune responses in the skin. It also is likely to have a pathophysiological role in the development of inflammatory skin diseases as highlighted by the recent successful use of biologics targeting TNF for the treatment of diseases such as psoriasis[315]. It is secreted by a variety of cells in including monocytes and

macrophages, T cells, fibroblasts and endothelial cells. Increased serum levels of TNF have been consistently reported in old mice and humans[316;317], seen in association with increased TNF production by leukocytes after LPS stimulation *in vitro*[318]. It is therefore of interest that in our study reduced quantities of TNF-α were produced at the site of Candin injection in the old. It has been proposed that increased levels of TNF production may be beneficial, by compensating for reduced IL-2 production in the old[319]. However, circulating TNF induces a catabolic state and has been shown to be associated with increased frailty in the old in addition to increased risk of diseases such as dementia, coronary vascular disease and atherosclerosis[316]. Studies investigating serum cytokine levels during acute pneumonia however have found reduced levels of TNF-α in addition to IL-8 and MIP 1α in the old[320], indicating an impaired production of TNF in response to pathogens in the old.

TNF is also required for the maturation of DC and has been shown to have a role in promoting the differentiation of CD14+ monocytes into cells with dendritic cell phenotype[321]. Injection of TNF-α intradermally in to humans has been shown to induce the migration of Langerhans cells from the epidermis and this has been shown to be impaired in the old[238]. In mouse studies, reduced migration to lymph nodes has been shown in ageing mice following TNF injection[322]. Studies on monocytes/macrophages and whole blood have shown reduced production of TNF-α after stimulation with LPS in old humans[320] and mice[323]. In addition, a recent study has shown reduced production of TNF by macrophages following stimulation *in vitro* with Candida antigen in mice[324]. These studies indicate that there may be an impaired production of TNF-α by monocytes and macrophages with ageing. Reduced production of TNF-α by monocytes in our old group may therefore explain the observed reduction in immune responses. The effects of ageing on monocytes, macrophages and dendritic cells in our old and young groups are investigated in Chapter 6.

The observed reduction in clinical response in the old may also be explained by a reduction in TNF-α levels in the old. TNF is thought to induce both vasodilatation and increased capillary permeability contributing to both the degree of erythema and induration at the site of the immune response respectively. Both of these parameters are included in the clinical score used in our model. In the young and old groups however, levels of TNF did not correlate with the clinical score observed suggesting that additional factors are involved in producing the observed clinical response in the skin. TNF also stimulates endothelial cells to increased expression of E-selectin, ICAM-1 and VCAM[101], promoting leukocyte adherence and therefore reduction in TNF production in old may explain reduced recruitment of lymphocytes in to the skin.

TNF also has anti-inflammatory effects and it has been proposed that TNF may have an important role in limiting tissue damage during systemic infection. TNF receptors belong to a superfamily of death receptors and are thought to induce apoptosis in keratinocytes and leukocytes during cutaneous inflammation[325;326]. Previous studies have shown increased sensitivity in the old to TNF induced T cell apoptosis[325;327], indicating that any TNF present at the site may have more effective anti-inflammatory effects in the old compared with the young, reducing the inflammatory response in the skin. The inflammatory effects of TNF can be limited by the production of soluble receptors that compete for binding of TNF on cell surfaces. Increased production of TNF receptors has been shown in the old[328] and may also account for the reduced measured levels of TNF in the old group in this study.

Following initiation of the immune response in the skin the production of chemokines/cytokines such as MCP-1, MIP-1α, RANTES and IL8 by various cell types is then crucial in order to allow for amplification of the response by recruitment of cells to the site and also local activation of cells. (Table 1.2 in introduction.) The production of these mediators is thought to be dependent on the activation of various cell types by IFN-γ and TNF-α[329]. MCP-1, MIP-1α, RANTES and IL8 can all be produced by monocytes upon activation and also

serve to regulate their responses[330]. However, resident skin cells also play a significant role in the production of chemokines in order to mediate T lymphocyte recruitment during inflammation. Activated keratinocytes, endothelial cells and dendritic cells have also been shown to produce IP-10, MCP-1, IL-8 and RANTES. In addition, dendritic cells have also been shown to produce MIP-1α and fibroblasts MCP-1[385].

A significant reduction in the levels of MCP-1, MIP-1α, RANTES and IL8 was found in the old compared with the young in this study, potentially resulting in a reduction in activation and recruitment of monocytes/macrophages, neutrophils [331]and lymphocytes[332-335] during immune responses in the old group. The reduced production of these mediators may be reflective of the observed reduced levels of IFN-γ[336;337] and TNF-α found in this study. However, previous studies have indicated an increased production of MIP-1α, RANTES from peripheral blood monocytes and T lymphocytes *in vitro*[337;338] in the old, highlighting the need for *in vivo* investigation of immune responses. The previously observed senescence of both fibroblasts and keratinocytes in old skin[339] may also explain in part the observed reduction in the production of chemokines in response to antigen injection.

6. Innate immunity and initiation of the immune response in the skin- the effects of ageing on macrophages and dendritic cells.

6.1 Introduction

Although a clinical response to the intradermal injection of antigen is only seen in those with prior immunity, suggesting that antigen-specific cell mediated response plays a key role in the DTH response to injection of antigen, the initial response to antigen in the skin may depend on non-specific immune responses via the innate immune pathway. Previous studies have indeed indicated that cellular infiltration into the skin during the course of the DTH response is biphasic with an initial infiltration of cells involved in innate immunity that occurs in non-sensitised subjects and then second specific peak seen only in those who have prior immunity to the antigen. Around 4-6 hours after antigen injection neutrophils are seen to infiltrate in to the skin, followed by macrophages that reach peak numbers at around 24 hours. It is only after 48 hours that the predominant cell type seen in the skin is the T lymphocyte[62;63].

The innate immune system plays an essential role in the first line of defence against pathogens. Ageing is associated with a breakdown in the epithelial barrier to pathogens provided by the skin, placing increased demands on the innate immune system in the old. Whilst in the past attention has focused on adaptive immunity as a cause for immunosenescence, recent work has indicated that cells of the innate immune system are also affected by advancing age including monocytes/macrophages, neutrophils, and NKT cells.

Our work has demonstrated a reduction in the numbers of lymphocytes present at the site of the DTH response from early time-points, in addition to a reduction in the concentrations of the acute inflammatory mediators TNF-α and IFN-γ, suggestive of a defect in the initiation of the immune response in the old. In particular the reduction of cytokines/ chemokines TNF, MCP-1, MIP 1α in blister fluid may indicate potential defects in monocyte/macrophage function as these factors are both produced by monocytes but also have an important role to play in their activation[385] . Monocytes mediate linkage between innate and adaptive immune system. While they act as crucial effector cells of the innate immune system, mediating the generation of inflammation, following the engulfment of foreign bodies they are also able to act as APCs in the context of MHC. Thus, a defect in monocytes with ageing may result in both reduced innate responses and a subsequent reduction in adaptive immune responses.

Dendritic cells also have an important role to play in the coordination of innate and adaptive immune responses. In normal, uninflammed skin, two main types of dendritic cell are observed; epidermal DCs, known as Langerhans cells, and dermal dendritic cells. DCs within the skin participate in the generation of a rapid innate immune response by reacting to invading pathogens and generating pro-inflammatory cytokines. Unlike macrophages, DCs are also uniquely able to stimulate naïve T cells and are essential for primary immune responses to antigens and the development of immune memory responses.

According to the classical model of the DTH response, DCs also play a role in the initiation of the secondary immune memory responses. After processing antigen, they are thought to migrate to the draining lymph node and stimulate the proliferation of effector memory T cells that subsequently migrate to the site of inflammation in the skin, resulting in the accumulation of antigen-specific T lymphocytes at the site of immune challenge. The observation that there is a large population of effector memory T cells within the skin with diverse receptor repertoire suggests however that local presentation of

antigen within the skin may be sufficient to initiate secondary immune responses in the skin[79;80]. Ageing has been shown to have various effects on dendritic cells including reduced phagocytosis and antigen presenting function. In the skin the effects of ageing, in particular photoageing, on Langerhans cells has been widely reported. Currently, however, available literature on the role of dermal dendritic cells and the effects of ageing on this is very limited.

In this chapter we compared the numbers and function of macrophage and dendritic cells following Candin injection in the young and old.

6.2 Proportions of cell types present at early time points in young and old.

In order to establish what proportions of cells were seen at an early time point in the young and old, skin suction blisters were induced at 24 hours after injection of candida antigen in 3 old and 3 young volunteers. Blister cells were analysed using flow cytometry and the proportions of cells were calculated according to the forward and side-scatter profiles of the cells (Figure 6.5) Of interest, there was a reduced proportion of neutrophils in the old (Figure 6.1), indicating that there may be defective recruitment of neutrophils to the site. This did not reach statistical significance.

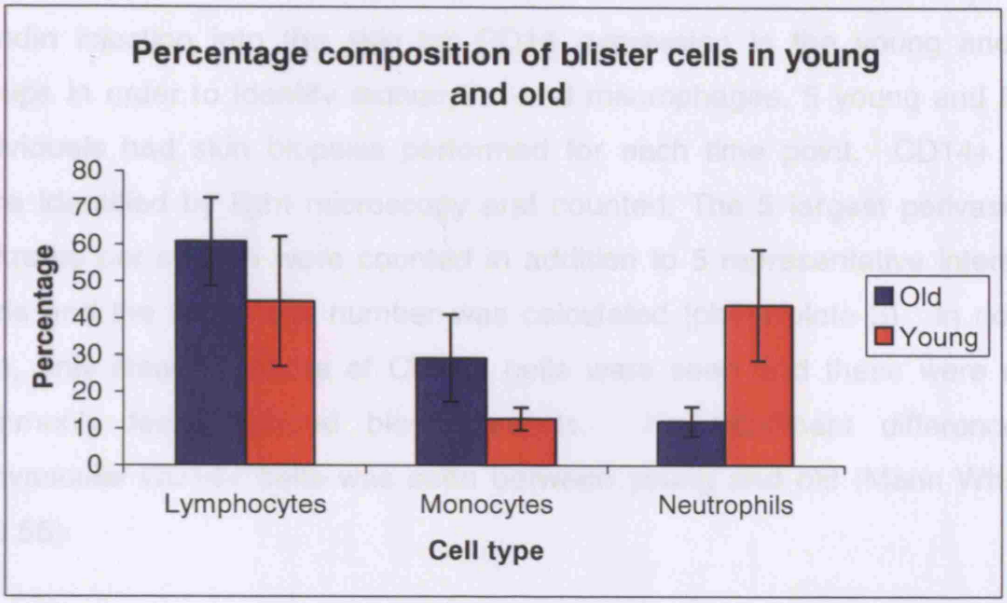


FIGURE 6.1 Percentages of lymphocytes, monocytes and neutrophils present in the SB cell population in young and old.
Percentages of different cell types present in skin suction blister fluid were determined using FSC/ SSC profiles on flow cytometry (Figure 6.5). Blisters were induced at 24 hours after Candin injection in to 3 young and 3 old individuals. A slight decrease in the percentage of neutrophils within the blister leukocyte cell population was observed, although this did not reach statistical significance (Mann Whitney p=0.1)

6.3 Monocyte and macrophage cell numbers within the skin

In view of the reduction in concentration of chemokines in the skin in the old thought to be important for the attraction of monocytes in to the skin, we next decided to investigate whether the observed reduction in chemokine concentration did indeed result in a reduction of numbers of monocytes and macrophages present within skin biopsies from old individuals taken at different time-points after the injection of Candin into the skin using immunohistological staining for common monocyte and macrophage markers.

6.3.1 Numbers of CD14+ cells in young and old skin biopsies following Candin injection.

CD14 is commonly used for the immunohistological identification of macrophages within tissues such as the skin and is the common epitope expressed on both monocytes and macrophages but not DCs. CD14 acts as a receptor for microbial antigens such as LPS in addition to yeasts such as

candida. We therefore stained skin biopsies taken at 0, 1, 3 and 7 days after Candin injection into the skin for CD14 expression in the young and old groups in order to identify monocytes and macrophages. 5 young and 5 old individuals had skin biopsies performed for each time point. CD14+ cells were identified by light microscopy and counted. The 5 largest perivascular infiltrates per section were counted in addition to 5 representative interstitial fields and the mean cell number was calculated (photo plate 3). In normal skin, only small numbers of CD14+ cells were seen and these were most commonly found around blood vessels. No significant difference in perivascular CD14+ cells was seen between young and old (Mann Whitney $p=0.55$).

In both young and old groups, an increase in the number of CD14+ cells was seen in both dermal interstitial and perivascular tissue at 24 hours (Figure 6.2 A+B). No significant difference was found between the number of CD14+ cells in the young and old at the 24 hour time point (Mann Whitney $p=1$ interstitial cells, Mann Whitney $p=0.3$ perivascular cells). In the young, there was then a decline in interstitial CD14+ cells at Day 3 (Mean: 24.93 cells at 24 hours, 6.53 cells at Day 3) (Figure 6.2A) with no further change in cell numbers at Day 7. In the old, although there was a small decrease in cell numbers at Day 3 and an apparent increase in cell numbers at Day 7 in the old, although this did not reach statistical significance (Wilcoxon ranked pairs test $p>0.5$)).

Perivascular CD14+ cells were then counted for biopsies taken each timepoint (Figure 6.2B). At 24 hours, there was an increase in the numbers of perivascular CD14+ cells in both the young and the old. Numbers of perivascular cells appeared to reach a peak at Day 3, declining at Day 7 although differences in cell numbers between these timepoints failed to reach statistical significance (Wilcoxon ranked pairs test $p>0.5$ for both young and old paired time points). No difference was found between old and young for all time points studied (Mann Whitney $p>0.5$ for all time points).

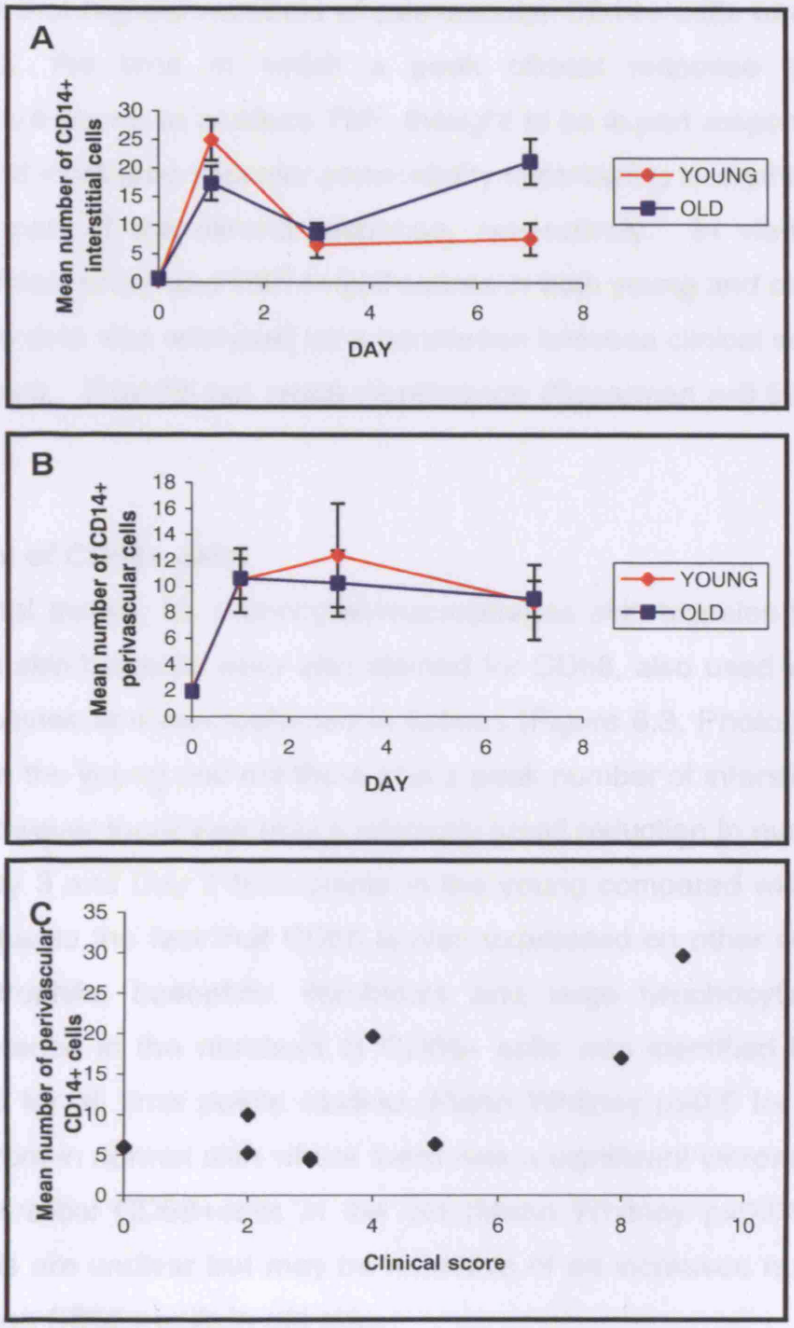


FIGURE 6.2 Numbers of interstitial and perivascular CD14+ cells in young and old skin biopsies following injection of Candin skin test solution.

Skin biopsies were taken from the inner aspect of the forearm of young and old individuals at various time points from 0-7 days at the site of injection of Candin skin test solution. A minimum of 5 individuals was tested in both young and old groups for all time points sampled. Fresh frozen sections were prepared from the skin biopsies and stained for CD14 using the indirect immunoperoxidase technique. For each section the mean cell count for either the 5 largest perivascular infiltrates or 5 representative interstitial fields was calculated. Error bars in A and B are representative of the SEM. A. Numbers of interstitial CD14+ cells. In both young and old interstitial cell numbers reached a peak at 24 hours, although in the old there appeared to be a further increase in cell numbers at Day 7. There was no significant difference in the numbers of cells between the 2 groups (Mann Whitney $p>0.5$) B. Perivascular CD14+ cell numbers reached their peak between days 1 and 3. No significant difference in cell numbers was found for any time point in the young and old. C. No correlation was found between the numbers of perivascular CD14+ cells at day 3 and the clinical score in both young and old individuals (Spearman $r=0.59$ $p=0.1$)

It is of interest that highest numbers of perivascular CD14+ cells were found around day 3, the time at which a peak clinical response is seen. Macrophages are known to produce TNF, thought to be in part responsible for vasodilation and increased vascular permeability contributing to erythema and induration as part of the clinical response, respectively. In view of the variability in clinical score and CD14+ cell counts in both young and old at Day 3 in the old, the data was analysed for a correlation between clinical score and CD14+ cell count. This did not reach significance (Spearman r=0.59, p=0.1) (Figure 6.2B).

6.3.2 Numbers of CD68+ cells

As an additional marker for monocytes/macrophages skin biopsies from the young and old skin biopsies were also stained for CD68, also used widely to stain for monocytes and macrophages in tissues (Figure 6.3, Photo plate 3). As for CD14, in the young and old there was a peak number of interstitial cells at 24 hours, however there was only a relatively small reduction in numbers of cells at the Day 3 and Day 7 time points in the young compared with CD14. This may be due to the fact that CD68 is also expressed on other cell types including neutrophils, basophils, fibroblasts and large lymphocytes. No statistical difference in the numbers of CD68+ cells was identified between young and old for all time points studied (Mann Whitney $p>0.5$ for all time points) apart from in normal skin where there was a significant increase in the number of interstitial CD68+cells in the old (Mann Whitney p=0.01). The reasons for this are unclear but may be reflective of an increased number of non-macrophage CD68+ cells in old skin.

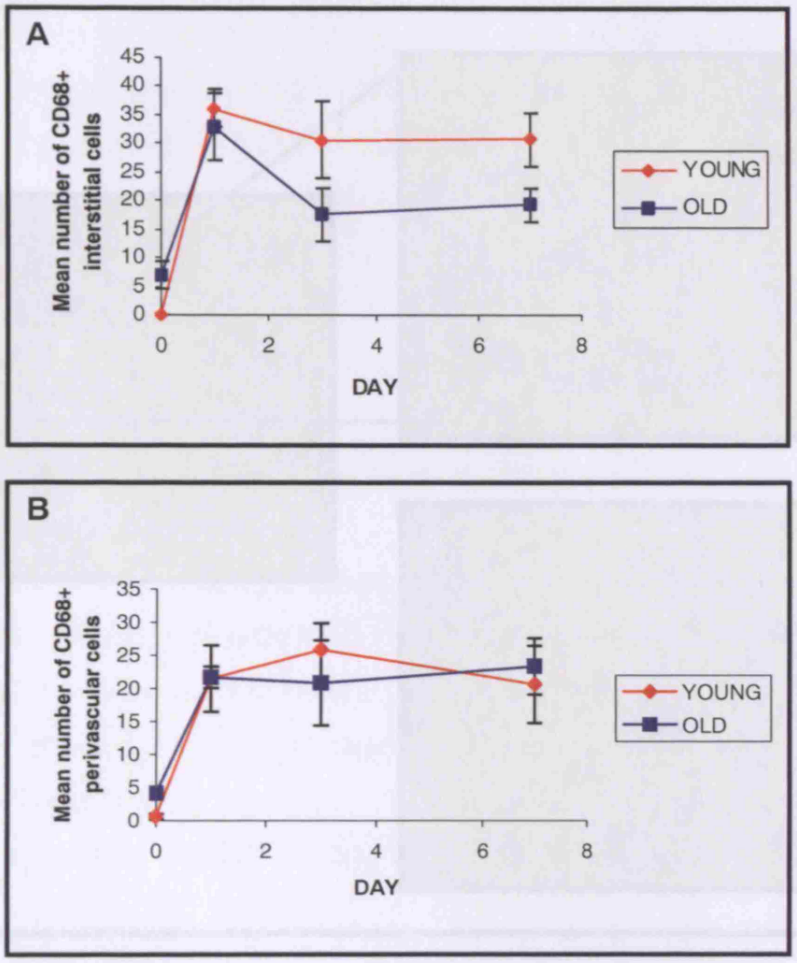
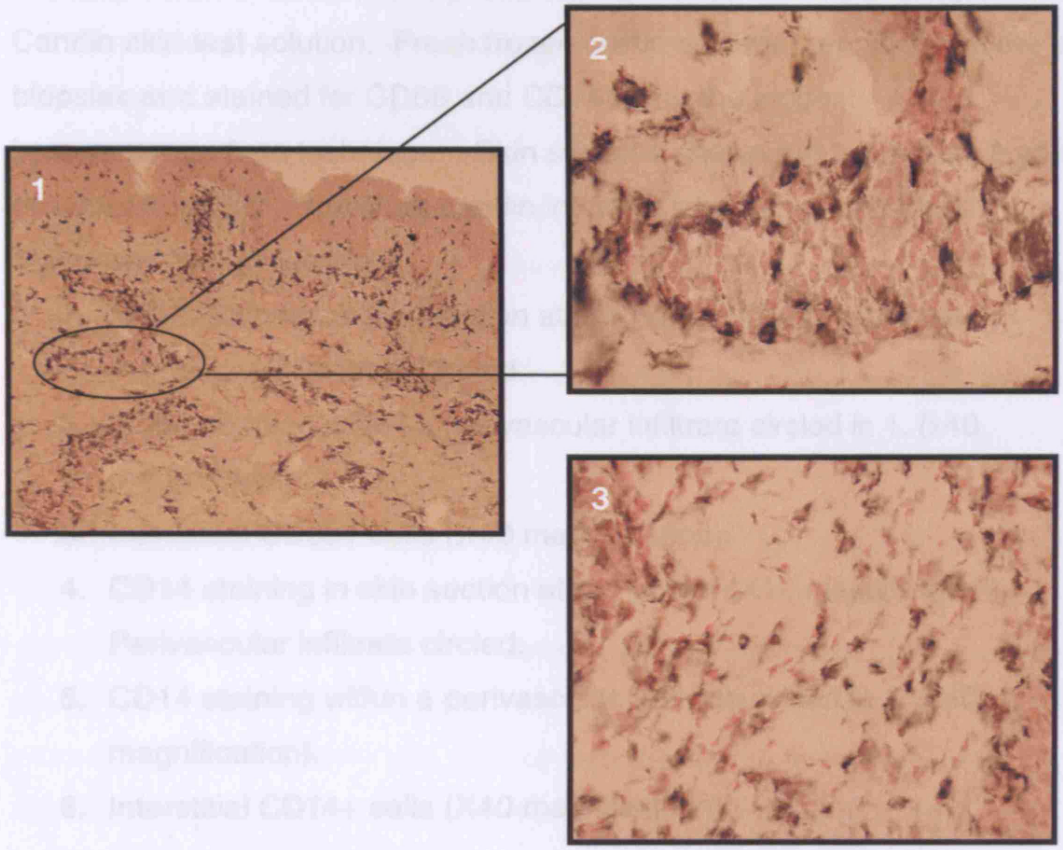


FIGURE 6.3 Numbers of interstitial and perivascular CD68+ cells in young and old skin biopsies following injection of Candin skin test solution.

Skin biopsies were taken from the inner aspect of the forearm of young and old individuals at various time points from 0-7 days at the site of injection of Candin skin test solution. A minimum of 5 individuals was tested in both young and old groups for all time points sampled. Fresh frozen sections were prepared from the skin biopsies and stained for CD68 using the indirect immunoperoxidase technique. For each section the mean cell count for either the 5 largest perivascular infiltrates or 5 representative interstitial fields was calculated. Error bars in A and B are representative of the SEM. A. Numbers of interstitial CD68+ cells. In both young and old interstitial cell numbers reached a peak at 24 hours. No significant difference in cell numbers was found between the young and old groups. B. Numbers of perivascular CD68+ cells. Numbers of cells reached a peak at around 3 days. No significant difference in cell numbers was found between the young and old groups.

PHOTO PLATE 3

CD68



CD14

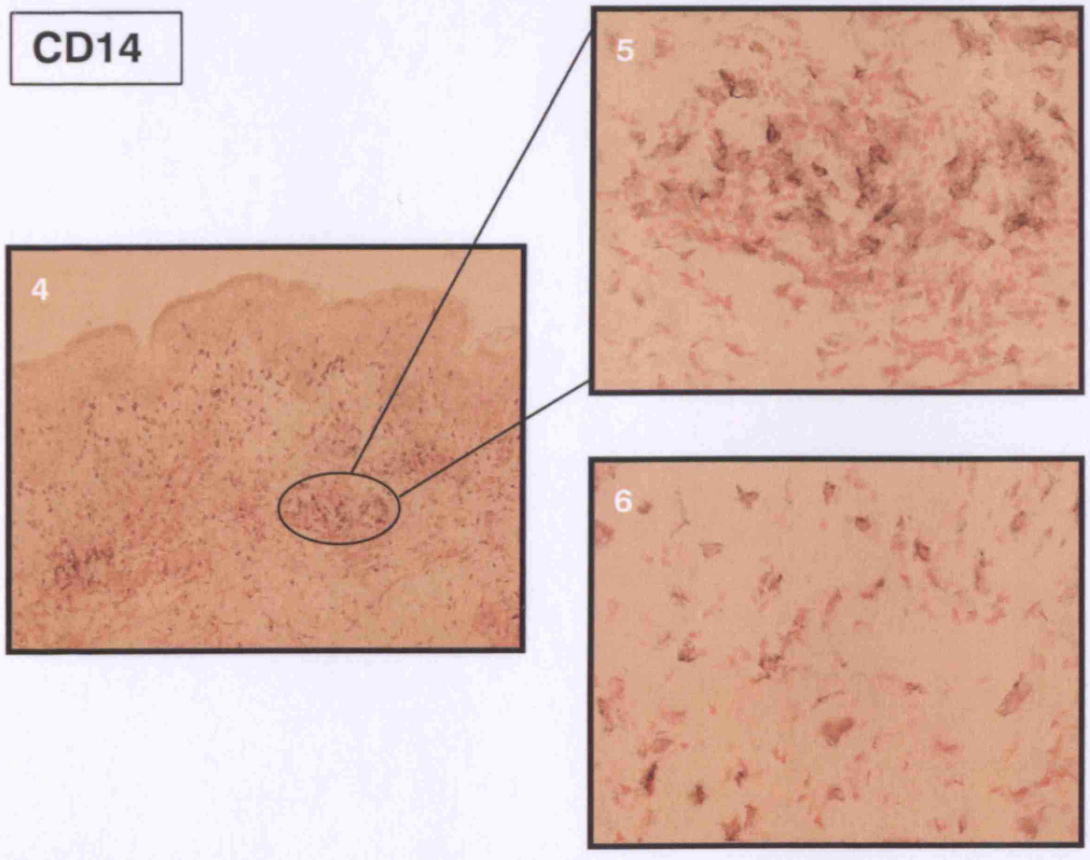


PHOTO PLATE 3. Skin biopsies stained for CD14+ and CD68+ cells at Day 3.

Skin biopsies were taken from the inner aspect of the forearm of young and old individuals at various time points from 0-7 days at the site of injection of Candin skin test solution. Fresh frozen sections were prepared from the skin biopsies and stained for CD68 and CD14 using the indirect immunoperoxidase technique. Skin sections shown are taken from 2 young individuals at Day 3 following candin injection who had good clinical responses to skin testing.

1. CD68 staining in skin section at low power (X10 magnification). Perivascular infiltrate circled.
2. CD68 staining within a perivascular infiltrate circled in 1. (x40 magnification).
3. Interstitial CD68+ cells (X40 magnification).
4. CD14 staining in skin section at low power (X10 magnification). Perivascular infiltrate circled.
5. CD14 staining within a perivascular infiltrate circled in 1. (x40 magnification).
6. Interstitial CD14+ cells (X40 magnification).

6.4 Activation of CD14+ monocytes at the site of the immune response

6.4.1 Expression of HLA DR, CD40, CD80, CD86 on CD14+ monocytes

The reduced concentration of TNF, MCP-1 and MIP1 may also result in reduced monocyte activation. Monocytes are known to upregulate several surface receptors upon activation including HLA DR and the costimulatory receptors CD40, CD80 and CD86. In order to assess whether in the old there is reduced activation of monocytes, suction blisters were induced at 24 hours after Candin injection. The monocyte population within the blister cells was then identified on the basis of forward/side scatter profile in addition to the expression of CD14 on flow cytometric analysis. (Figure 6.5). Cells were also stained with HLA-DR, CD40, CD80 and CD86 and the MFI and percentage positive gated cell population was calculated for each receptor (Figure 6.5).

In both young and old individuals, the expression of HLA DR, CD40, CD80 and CD86 on CD14+CD4+ monocytes derived from blister cells was significantly higher than in the blood-derived population (Wilcoxon ranked pairs test $P<0.05$ for all receptors) (Figure 6.4), indicating activation of monocytes derived from the skin at the site of the immune response. No significant difference in receptor expression was found between young and old (Mann Whitney $p>0.05$ for all receptors) respectively.

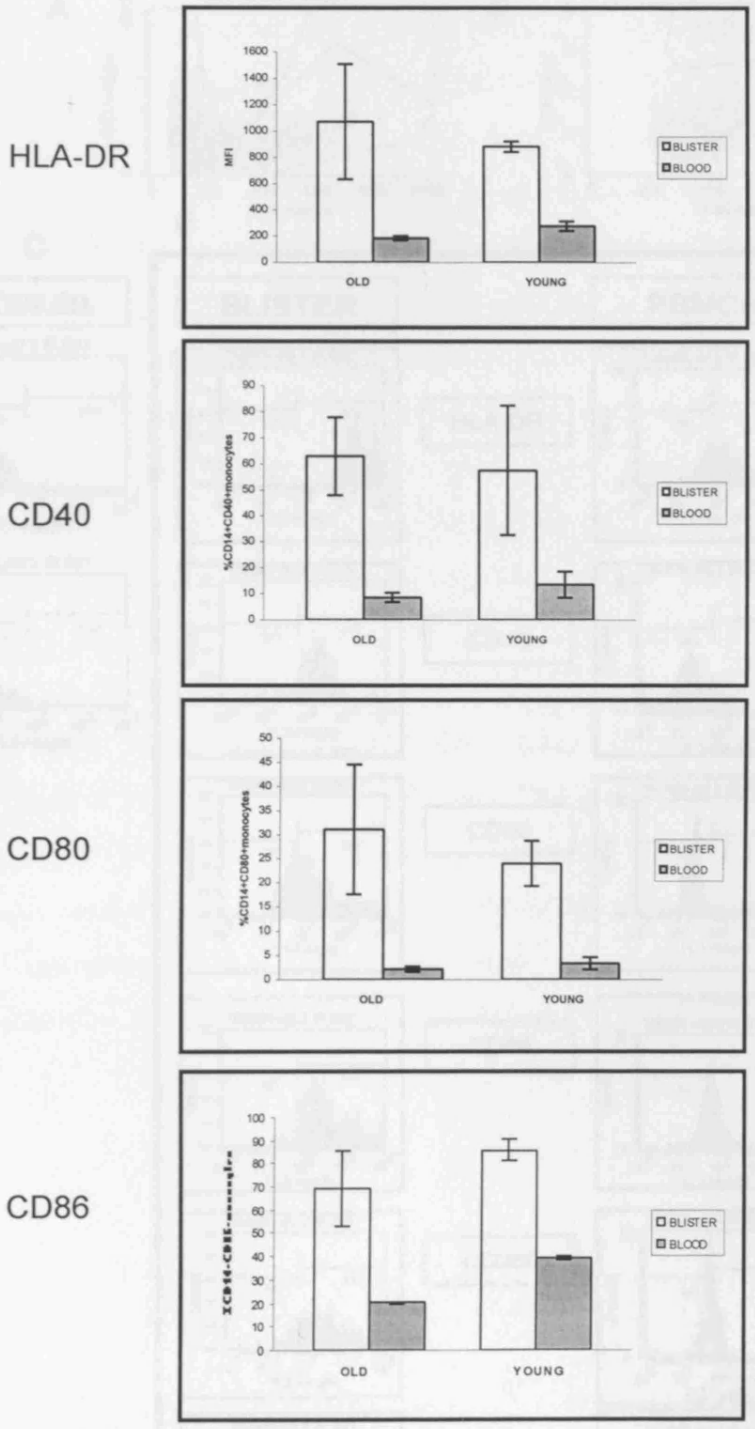


FIGURE 6.4 Expression of surface markers on CD14+ monocytes isolated from peripheral blood (PBMCs) and skin suction blisters induced following Candin injection Expression of receptors on CD14+ monocytes isolated from skin suction blisters and PBMCs at 24 hours following the injection of Candin skin test solution in 3 old and 3 young volunteers. Skin suction blister cells were stained with antibodies to CD14 in addition to either HLA-DR, CD40, CD80 or CD86 and analysed by flow cytometry. The CD14+ monocyte population was identified on the basis of forward scatter and staining with CD14 (Figure 6.5). Cell populations with positive staining for the different receptors were then identified within the CD14+ monocyte population.

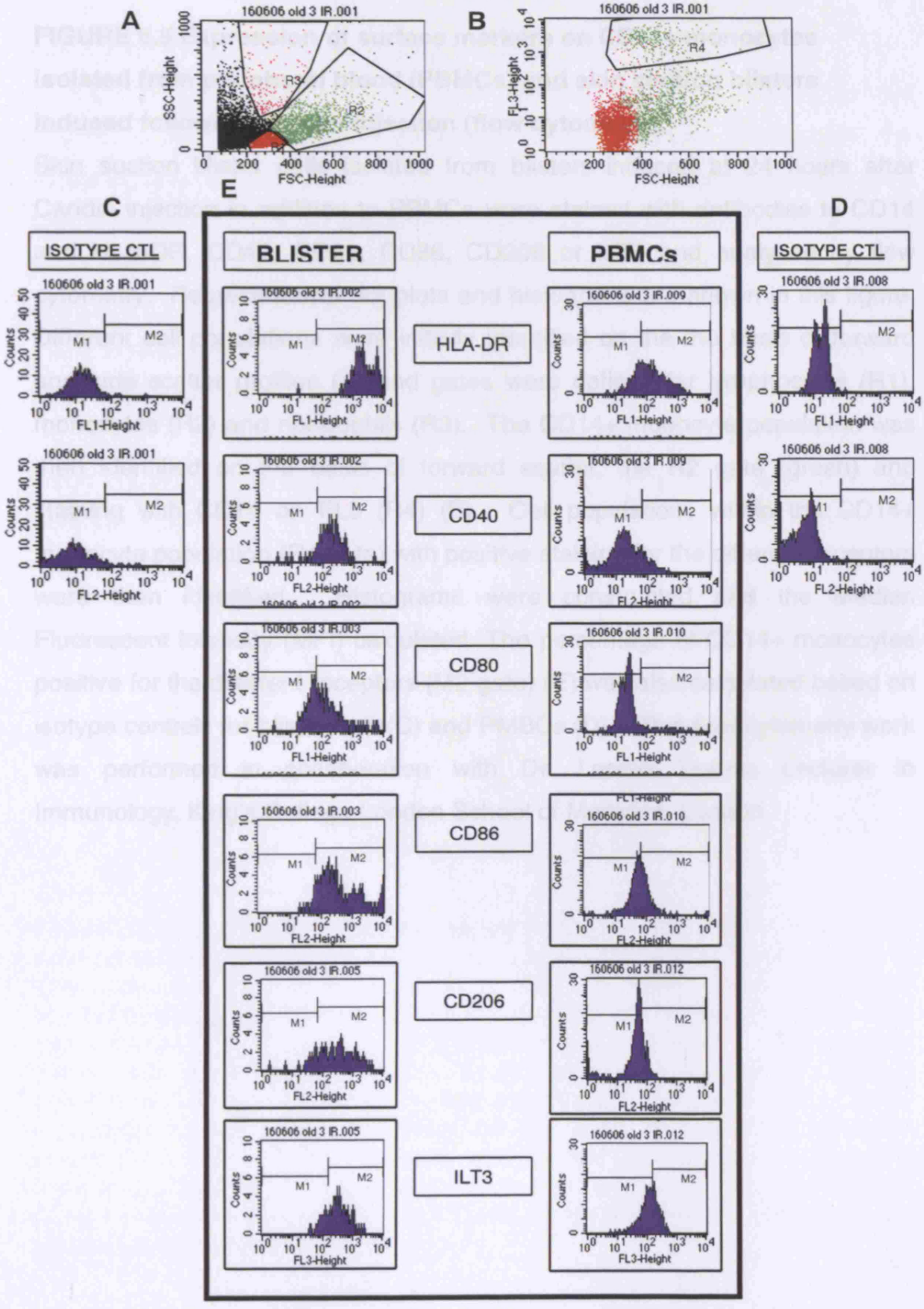


FIGURE 6.5 Expression of surface markers on CD14+ monocytes isolated from peripheral blood (PBMCs) and skin suction blisters induced following Candin injection (flow cytometry).

FIGURE 6.5 Expression of surface markers on CD14+ monocytes isolated from peripheral blood (PBMCs) and skin suction blisters induced following Candin injection (flow cytometry).

Skin suction blister cells isolated from blisters induced at 24 hours after Candin injection in addition to PBMCs were stained with antibodies to CD14 and HLA-DR, CD40, CD80, CD86, CD206 or ILT3 and analysed by flow cytometry. Representative dot plots and histograms are shown in this figure. Different cell populations were initially identified on the the basis of forward and side scatter profiles (A) and gates were defined for lymphocytes (R1), monocytes (R2) and neutrophils (R3). The CD14+ monocyte population was then identified on the basis of forward scatter, the R2 gate (green) and staining with CD14 on FL3 (R4) (B). Cell populations within the CD14+ monocyte population (R4 gate) with positive staining for the different receptors were then identified. Histograms were constructed and the Median Fluorescent Intensity (MFI) calculated. The percentage of CD14+ monocytes positive for the different receptors (M2 gate) (E) was also calculated based on isotype controls for blister cells(C) and PMBCs (D). This flow cytometry work was performed in collaboration with Dr. Leonie Taams, Lecturer in Immunology, King's College London School of Medicine, London.

6.4.2 Expression of the inhibitory receptor ILT3

In addition to pro-inflammatory effects, certain groups of monocytes are known to have tolerogenic properties including those expressing the Immunoglobulin- like transcript 3 (ILT3) receptor[340]. We therefore investigated the expression of ILT3 on CD14+CD4+ monocytes (Figure 6.6). No significant difference was found for skin suction blister derived CD14+ monocytes between young and old groups (Mann Whitney p=1), suggesting that the reduced clinical and cellular response in the old is not secondary to an active suppression by monocytes. However, there was an increase in expression of ILT3 in skin- derived monocytes compared with the peripheral blood in both the young and old groups (Wilcoxon ranked pair test p=0.03 for both groups).

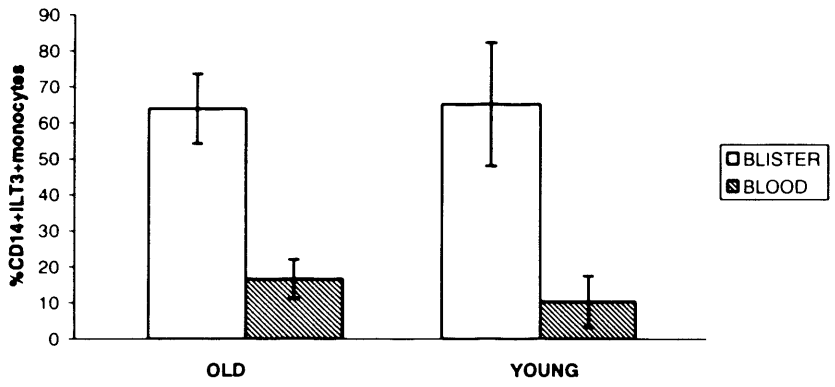


FIGURE 6.6 Expression of ILT3 receptor in CD14+ monocytes on skin suction blisters and PBMCs at 24 hours after injection of Candin.
The expression of ILT3 receptor on CD14+ monocytes isolated from skin suction blisters and PBMCs at 24 hours following the injection of Candin skin test solution in 3 old and 3 young volunteers was assessed. Skin suction blister cells were stained with fluorescent labelled antibodies to CD14 in addition to ILT3 and analysed by flow cytometry (Figure 6.5). The CD14+ monocyte population was identified on the basis of forward scatter and staining with CD14. Cell populations with positive staining for ILT3 receptors were then identified within the CD14+ monocyte population. No difference in CD14+ monocyte expression of ILT3 was found between the young and old groups (Mann Whitney p=1)

6.4.3 Expression of the mannose binding lectin protein receptor CD206

Previous studies have identified a reduction in the phagocytic capacity of monocytes in the old[226]. The expression of the mannose binding receptor CD206 is thought to correlate with the phagocytic capacity of monocytes and is upregulated on activated cells[341]. Comparison of CD206 expression on CD4+CD14+ monocytes in young and old did not reveal any significant difference between the two groups (Mann Whitney p=0.5) (Figure 6.7).

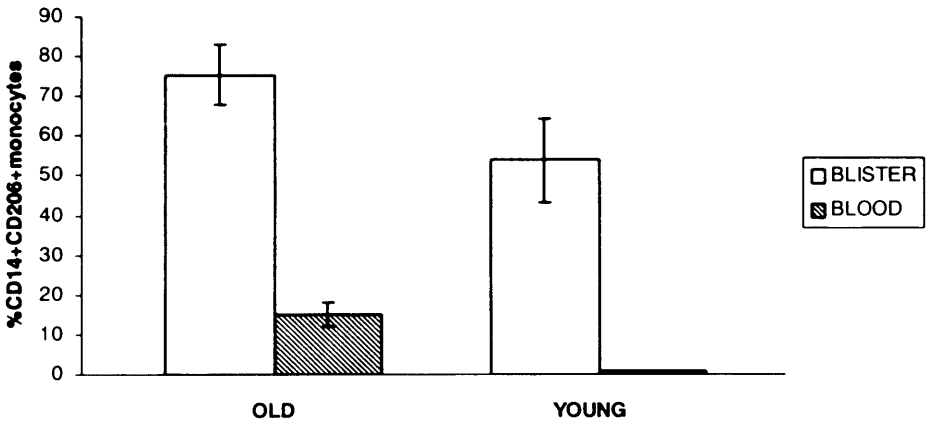


FIGURE 6.7 Expression of CD206 receptor in CD14+ monocytes on skin suction blisters and PBMCs at 24 hours after injection of Candin.

Expression of CD206 receptor on CD14+ monocytes isolated from skin suction blisters and PBMCs at 24 hours following the injection of Candin skin test solution was studied in 3 old and 3 young volunteers. Skin suction blister cells were stained with fluorescent labelled antibodies to CD14 in addition to CD206 and analysed by flow cytometry (Figure 6.5). The CD14+ monocyte population was identified on the basis of forward scatter and staining with CD14. Cell populations with positive staining for CD206 receptors were then identified within the CD14+ monocyte population.

6.5 Dendritic cells

6.5.1 CD1a expression

CD1a is expressed on Langerhans cells within the epidermis and also a small subset of dermal dendritic cells within the dermis. CD1 proteins bind to lipids to form antigen complexes that contact T cell receptors and activate T cells[342] Biopsies of normal skin from the forearm of both young and old individuals were analysed for CD1a expression after staining with a CD1a monoclonal antibody, using the immunoperoxidase technique. Transverse sections of the skin revealed CD1a+ dendritic shaped cells predominantly in the basal and suprabasal levels of the epidermis in both young and old individuals, consistent with Langerhans cells. (Photo plate 4). CD1a+ dendritic cell numbers were counted within the epidermis and the number of cells per unit area was calculated. No significant difference in the number of CD1a positive cells per unit area was found between young and old group (Mann Whitney $p=0.28$), however thinning of the epidermis with reduced rete ridges was observed in several individuals the old group (Photo plate 4 (3)) and thus it is possible that the actual total numbers of cells may have been reduced in the old. Normal skin also contained very small numbers of CD1a positive cells mainly around blood vessels in the young and old. These cells had less prominent dendrites (Photo plate 4 (2)) and therefore may be reflective of either migrating Langerhans cells with altered morphology compared with their epidermal counterparts or dermal dendritic cells.

Following injection of intradermal Candin, increased levels of CD1a positive cells were observed within the dermis at 24 hours in the young and old individuals (Photo plate 4(4+5)). CD1a cells were seen predominantly around blood vessels, although a few cells with dendritic morphology were also seen within interstitial areas. Unfortunately, the precise quantification of the numbers of CD1a+ cells around blood vessels was not possible due to lack of clarity of the staining. However, subjective qualitative analysis of stained skin biopsies indicated that in both the young and old there was an increase in dermal CD1a+ cells up to Day 7, the latest time point studied.

In the young group, CD1a+ cells were counted in the epidermis and the number of cells per unit area was calculated for normal skin in addition to biopsies taken at 1,3 and 7 days. No change in the numbers of CD1a+ cells per unit area could be detected between any of the time points (Kruskall Wallis test $p>0.5$).

PHOTO PLATE 4.

CD1a

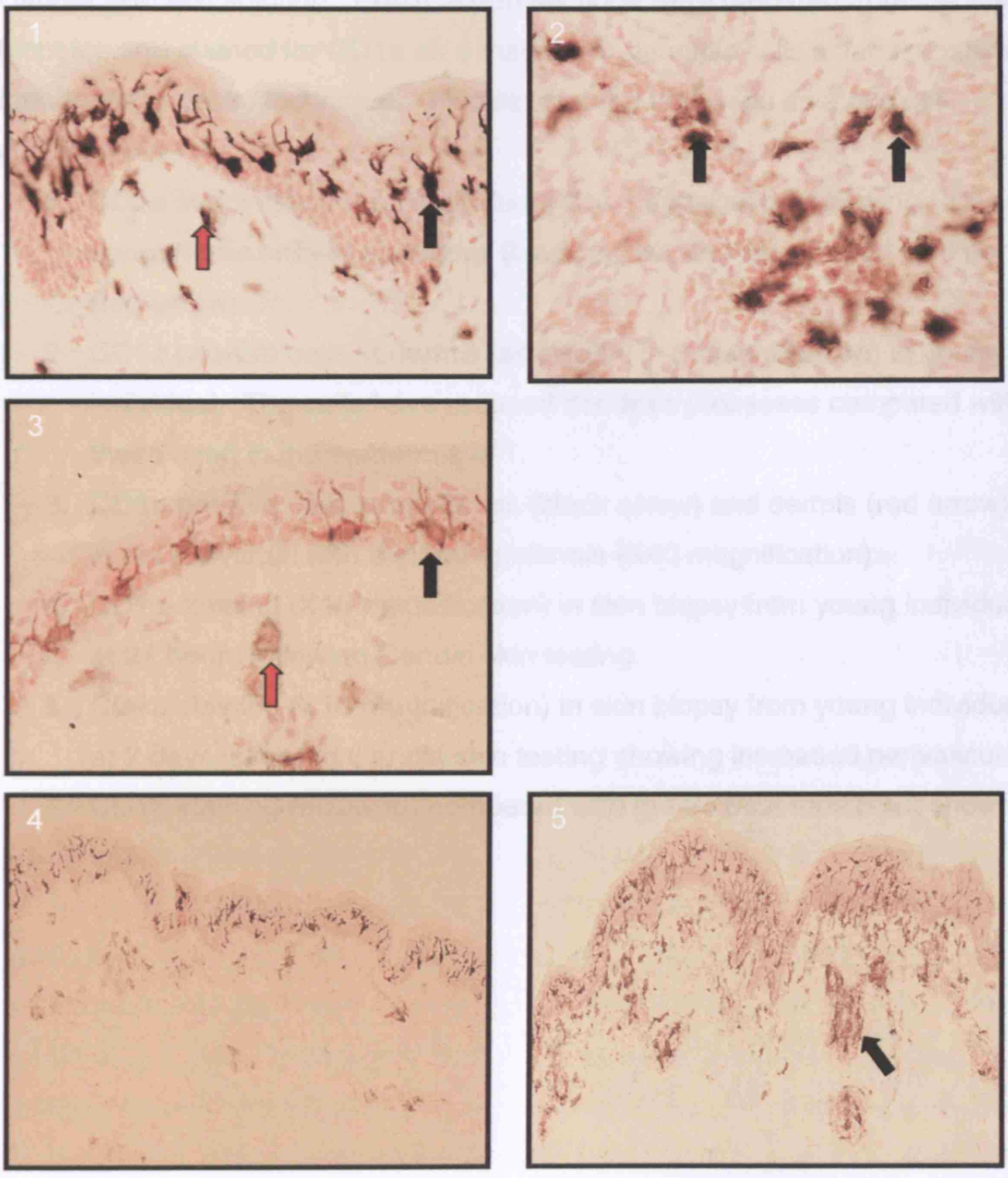


PHOTO PLATE 4. CD1a staining in young and old skin biopsies

Skin biopsies were taken from the inner aspect of the forearm of young and old individuals at various time points from 0-7 days at the site of injection of Candin skin test solution. Fresh frozen sections were prepared from the skin biopsies and stained for CD1a as a marker for dendritic cells using the indirect immunoperoxidase technique. Nuclear fast red was used as a nuclear counterstain.

1. CD1a staining in young individual (X20 magnification) showing CD1a+ Langerhans cells in epidermis (black arrow) and dermal CD1a+ cells (red arrow).
2. CD1a positive cells in dermis (arrowed) (X40 magnification) in young individual. The cells have reduced dendritic processes compared with those seen in the epidermis in 1.
3. CD1a positive cells in epidermis (black arrow) and dermis (red arrow) in old individual with thinned epidermis (X40 magnification).
4. CD1a staining (X10 magnification) in skin biopsy from young individual at 24 hours following Candin skin testing.
5. CD1a staining (X10 magnification) in skin biopsy from young individual at 7 days following Candin skin testing showing increased perivascular CD1a staining (arrowed) compared with the 24 hour time point shown in 4.

6.5.2 HLA-DR expression

In order for antigen to be presented to T lymphocytes, the injected material such as PPD or Candin first needs to be ingested by dendritic cells and broken down small antigenic fragments. Upon digestion, small peptide fragments are bound to the peptide binding groove of MHC class II molecules (HLA-DR, HLA-DP and HLA-DQ) and are transported to the cell surface for display to CD4+ T helper cells. Within the skin, HLA-DR is expressed on both dendritic cell populations such as Langerhans cells and dermal dendritic cells in addition to activated macrophages, endothelial cells[74;343-345] and lymphocytes. Previous studies have shown that in inflammatory skin diseases keratinocytes can also be induced to express HLA-DR[346;347].

HLA-DR expression within the skin was investigated as an indication of the number of cells present in the skin with potential antigen-presenting capacity. A reduction in HLA-DR expression would potentially result in reduced antigen presentation to T cells in the old and explain both the reduced numbers of CD4+ T cells in addition to a reduction in the number of antigen –specific cells present.

In normal skin biopsies from young and old individuals, HLA-DR expression was found to be present in both the epidermis and around blood vessels in the papillary dermis (photo plate 5 (1)). Positively staining cells within the epidermis had cellular morphology consistent with that of Langerhans cells . No difference in the extent of HLA-DR staining in young and old skin could be identified. It was not possible to accurately quantify HLA-DR + cells in the dermis however a qualitative increase in HLA-DR expression predominantly around blood vessels occurred within the skin at 24 hours and this was found to have increased further at both Day 3 and Day 7 in both the young and old groups (Photo plate 5 (2+3)). There was a reduction in the expression of HLA-DR in old individuals who failed to develop any clinical response in the skin at both Days 3 and 7 (Photo plate 5 (4)).

PHOTO PLATE 5

HLA- DR

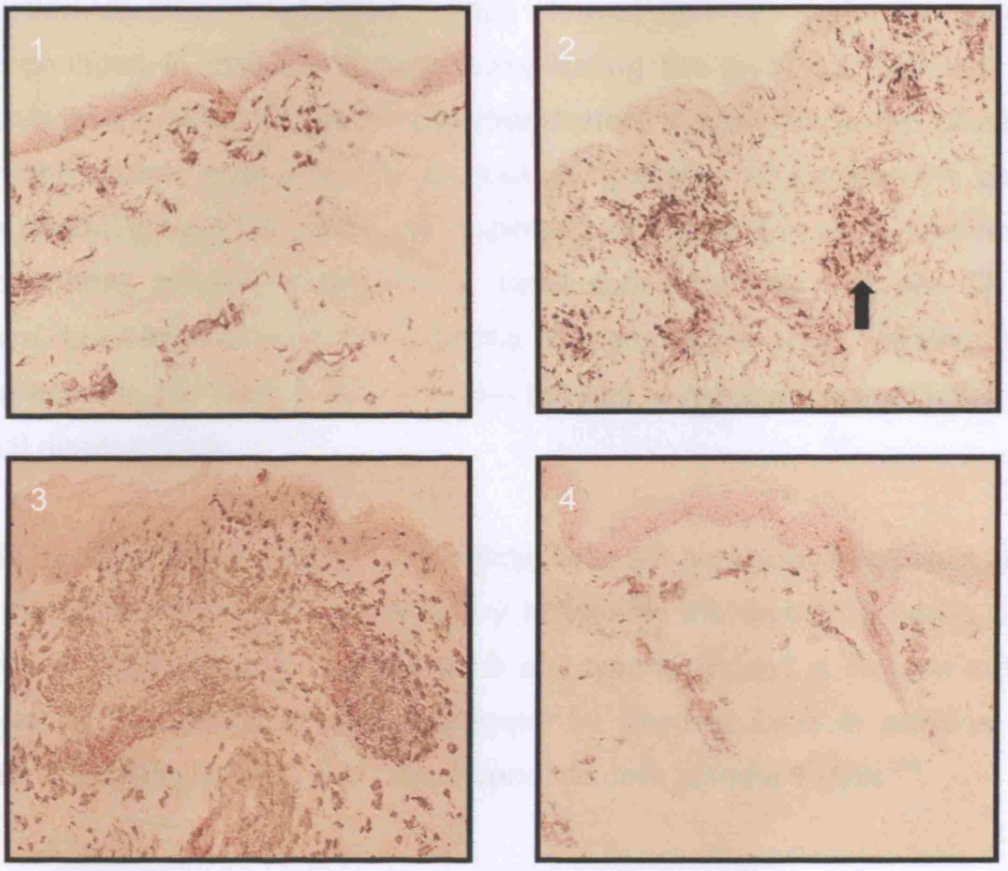


PHOTO PLATE 5 HLA-DR staining in young and old skin biopsies following Candin skin testing.

Skin biopsies were taken from the inner aspect of the forearm of young and old individuals at various time points from 0-7 days at the site of injection of Candin skin test solution. Fresh frozen sections were prepared from the skin biopsies and stained for HLA-DR using an indirect immunoperoxidase technique. Nuclear fast red was used as a nuclear counterstain.

1. HLA-DR staining in normal skin (X10)
2. HLA-DR staining in a skin biopsy taken at day 3 following Candin injection (X10) in a representative young individual, showing increased perivascular staining compared with normal skin (arrowed).
3. HLA-DR staining in a skin biopsy taken at day 7 (X10) following Candin injection in a representative young individual showing further increase in HLA-DR staining around perivascular infiltrates compared with days 3 and 7.
4. HLA-DR staining in an old individual at day 7 (X10) following Candin skin testing. The old individual had a poor clinical response to Candin injection in the skin. Reduced HLA-DR staining is seen compared with that seen in the young skin biopsy shown in 3.

6.5.3 Dermal dendritic cells: DC-SIGN expression

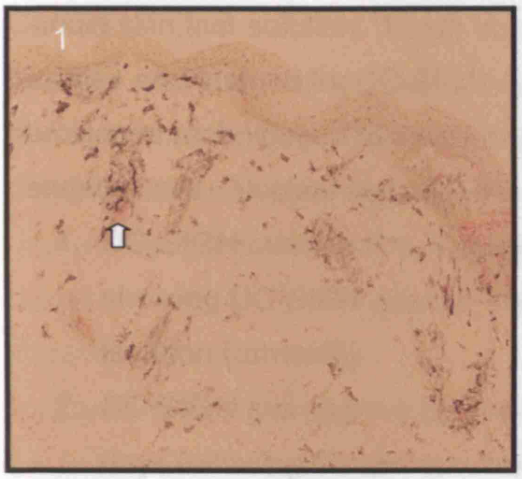
The dermis is known to contain a group of HLA-DR + dendritic cells known as dermal dendritic cells. Unlike the epidermis, which contains a homologous population of DCs (Langerhans cells), dermal dendritic cells are rather heterogeneous in their phenotype, complicating the study of their role in immunity in the skin. As we have demonstrated, a small population of cells within the dermis stain with CD1a, possibly indicative of Langerhans cells either migrating from the skin to the lymphatics or migrating in to the skin from the peripheral blood. In addition, a small subset of cells that are CD1a positive, but Langerin (a marker specific for Langerhans cells) negative are also present in the dermis and these are thought to represent a population of dermal dendritic cells.

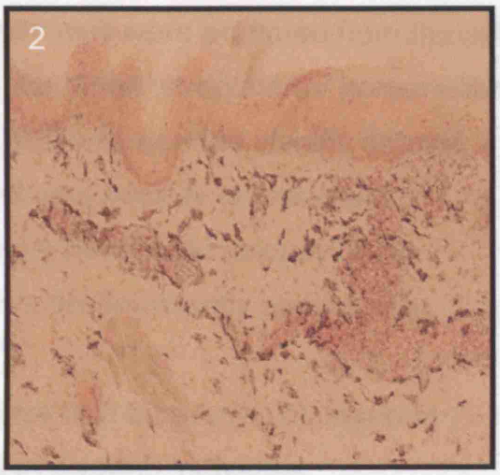
DC-SIGN is expressed on dermal dendritic cells but not Langerhans cells and was therefore used in this study to try to identify the dermal dendritic cell population within the skin. DC-SIGN is a C-type lectin and is thought to be involved in the internalization of antigens by dendritic cells in addition to contributing to the potency with which dendritic cells activate T cells[348].

Normal skin biopsies from both young and old individuals were found to contain DC-SIGN positive cells (Photo plate 6 (1)). As previously described, the cells were found within the papillary dermis predominantly around capillary vessels, although a few DC-SIGN+ cells were also seen within the interstitium (Photo plate 6). Although there was a subjective, small increase in DC-SIGN expression seen at 24 hours in DC-SIGN expression in both the young and old, expression of DC-SIGN was then found to remain relatively constant throughout the course of the immune response with similar numbers of positive cells at all time points studied. The DC-SIGN+ cells remained in a predominantly perivascular location, around the edges of the perivascular infiltrates (Photo plate 6 (2+3)).

PHOTO PLATE 6

DC-SIGN





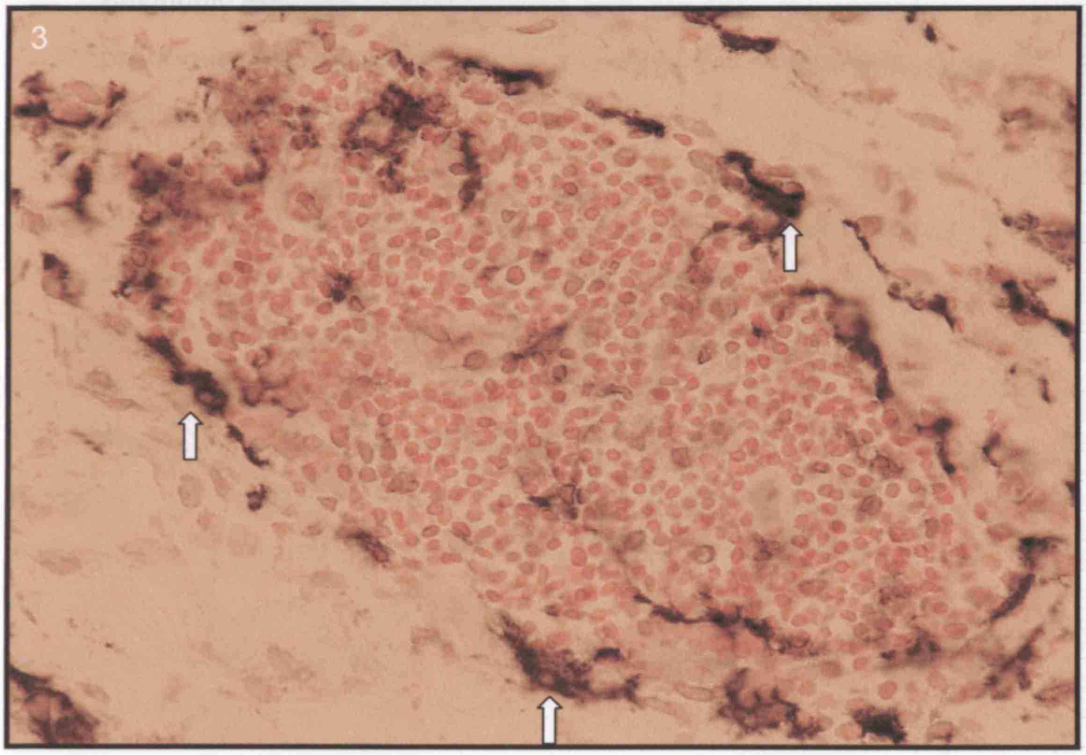


PHOTO PLATE 6. DC-SIGN staining in skin biopsies

Skin biopsies were taken from the inner aspect of the forearm of young and old individuals at various time points from 0-7 days at the site of injection of Candin skin test solution. Fresh frozen sections were prepared from the skin biopsies and stained for DC-SIGN using the biotin/ streptavidin/ horseradish peroxidase technique. DC-SIGN expression was used to identify dermal dendritic cells. Nuclear fast red was used as a nuclear counterstain.

1. DC-SIGN staining in a representative normal skin biopsy (X10), showing DC-SIGN positive cells in a predominantly perivascular location (arrowed).
2. DC-SIGN staining in a representative skin biopsy (X10), taken at 7 days following Candin skin testing in a young individual.
3. DC-SIGN positive cells in a perivascular infiltrate (X40) showing positively staining cells (arrowed) with dendritic morphology predominantly around the outer aspect of the cellular infiltration.

6.6 Discussion

Monocytes/ macrophages play a key role during the initiation of DTH responses in the skin. Previous studies have shown that monocytes start to appear within the skin at around 12 hours, reaching a peak at around 24-48 hours at which time they constitute around 80-90% of the cellular infiltrate[62;63]. These cells are likely to be important for the production of pro-inflammatory mediators, phagocytosis destruction of invading pathogens and antigen presentation to T lymphocytes within the skin. The finding that several key cytokines/ chemokines that are known to be produced by monocytes were reduced in our DTH model following candin injection (discussed in Chapter 5) led us to investigate whether a possible defect in monocytes/ macrophages within the skin in the old could explain the reduced immune responsiveness in ageing skin.

Firstly, the numbers of CD14+ and CD68+ cells within the skin in old and young skin biopsies following the injection of Candin were compared. Both of these markers are known to identify monocytes/macrophages, although neither are entirely specific for these populations of cells. No significant difference in the numbers of cells could be identified in the young and old groups. Previous studies in the old have however identified reduced migratory capacity of macrophages, in particular a reduction in macrophage chemotactic response to complement derived factors[226] although this conclusion was based on an *in vitro* assay rather than, as in this study, *in vivo* work.

As far as we are aware there have been no studies looking at the activation of monocytes within old skin during the course of a secondary immune response. Reduced production of monocyte- derived MIP-1α[227] and MCP-1[349] within the skin have however been described and has also been found in our model, implying that there may be reduced activation of monocytes/ macrophages within the skin. In addition, reduced production of various cytokines including IL-6 and TNF-α have been described for monocytes from peripheral blood, in addition to reduced MHC class II expression. We were

unable to identify any difference in the expression of a variety of activation markers on skin suction blisters in the young and old. However, only a limited number of samples were analysed. An unrepresentative old person who had a good clinical response to Candin injection possibly biased the results. Although no difference in the expression of HLA-DR was detected on CD14+ monocytes isolated from skin suction blisters, a potential overall decrease in MHC class II expression in old skin was seen within skin biopsies. It is of interest that an increase in HLA-DR expression has previously been reported in activated CD3+ T lymphocytes in the old[350]. The reduction in HLA-DR expression seen in our samples may be representative of an overall reduction in lymphocyte cell numbers in the old, a reduction in cellular activation or reduction in antigen-presenting cell expression of this marker.

Both murine models[224] and in elderly humans with chronic bronchitis [226]a reduction in the phagocytic capacity of macrophages has been identified. The mannose receptor CD206 is a type I transmembrance glycoprotein from a family of endocytic receptors that is expressed on macrophages in addition to fibroblasts and endothelial cells. Expression of CD206 on monocytes is thought to reflect their phagocytic capacity and is upregulated on activated cells. The increased expression of CD206 on monocytes in the skin compared with the blood in both the young and old indicates that monocytes recruited to the skin during the immune response are activated. No difference in CD206 expression on monocytes isolated from the young and old skin suction blisters could be identified. The role of phagocytosis promoting receptors and signal transduction components in ageing skin remain unknown.

The expression of the soluble Immunoglobulin- like transcript 3 (ILT3) receptor was also analysed on CD14+ monocytes. ILT3 is a cell surface molecule that is thought to be selectively expressed by myeloid antigen presenting cells such as monocytes, macrophages and dendritic cells[340] and is thought to render the cells tolerogenic by reducing their expression of co-stimulatory molecules and inducing antigen-specific unresponsiveness in CD4+ T helper cells[340;351]. ILT3 expression on monocytes is thought to be induced by a

subset of regulatory T cells known as CD8+CD28- T suppressor cells[352]. Although we were unable to identify any difference in the expression of ILT3 between young and old groups it is of interest that there was significantly higher expression of this receptor in the skin at the site of inflammation compared with peripheral blood. This indicates that there may be some role for this receptor in the control of immune responses in the skin

Skin biopsies taken from the young and old were also analysed for populations of antigen presenting cells at different time points following the injection of Candin. The DC system within the skin has great heterogeneity and complexity and the study of these populations is made difficult by overlapping expression of different cell surface markers. There are two main populations of DCs that occur in normal skin: epidermal langerhans cells characterized by the expression of Langerin/CD207 and DDCs in the dermis in humans characterized by the expression of DC-SIGN. CD1a was used as a marker for Langerhans cells in this study. CD1a is used commonly as a marker for Langerhans cells however a small subset of DDCs also express CD1a[353]. Dermal dendritic cells can also express CD14 used as a marker for monocytes in this study[353]. DC-SIGN distinguishes DDCs from other dermal populations although DC-SIGN can also be expressed by macrophages under certain conditions[354].

There is currently very little data on the relative contribution of different DC subsets in shaping the immune response. Further, although a significant amount of work on skin DCs has focused on LCs and migration in primary immune responses, in particular in response to the topical application of contact sensitizing agents, little data exists for role/migration of DCs in secondary immune responses in the skin. Although work on epidermal APCs such as Langerhans cells is likely to be relevant to the topical application of antigen, the investigation of dendritic cells derived within the dermis is equally important and it has been proposed that some skin diseases may begin in dermis via activation of DDCs[355]. Dermal dendritic cells have been demonstrated to be at least as potent as epidermal and peripheral blood derived DCs in their capacity to stimulate allogeneic resting T cells or

providing accessory function to autologous T cells activated with SEB or PHA[356]. Furthermore, DDCs have been shown to be more potent than monocytes in their antigen presenting capacity[356], suggesting that monocytes that are seen to infiltrate sites of DTH responses may not be the strongest APC population present in the skin.

Both Langerhans cells and dermal dendritic cells are derived from CD34+ myeloid progenitor cells within the bone marrow. In humans, the subsequent differentiation of these cells is based on *in vitro* studies. Several precursors, including CD14+ monocytes, that can differentiate in to LCs *in vitro* have been described in peripheral blood following exposure to combinations of GM-CSF, TGF-β, TNF-α, IL4 and IL13[357;358]. In addition, skin-resident CD14+ cells have been shown to acquire features of LC when cultured with TGF[359].

The majority of resident skin antigen presenting cells are present in immature state. Maturation of DCs is dependent on several signals including TNF production, T cell signals and stimulation of TLRs[360]. Maturation of DCs is necessary in order for them to change from immature antigen capturing cells to antigen presenting cells, allowing for development in adaptive immune responses. Although studies in mice have shown a reduction in TLR4 expression on dendritic cells in old mice[221], studies on monocyte derived DCs in humans has failed to confirm this finding[222]. Reduction in TNF levels seen in this study may also have an impact on DC maturation and may impact on ability to present antigen in skin in old.

Using transverse skin sections we were unable to determine any reduction in the number of CD1a cells within the epidermis during the course of the response. Previous studies have however reported a reduction of around 20% of cells following either trauma or injection of cytokines TNF-α and IL-1β into the skin. Reduced migration of LCs in response to TNF have also been demonstrated in the old, however there was only a slight reduction and it is not clear whether there is redundancy, whether this relates to the intradermal

injection of an antigen or whether LC migration is in fact important for the development of a DTH response in the skin.

Traditionally, epidermal sheets have been used for the analysis of numbers of Langerhans cells within the epidermis as this enables more accurate identification of individual cells. Although in our study only cell bodies and not dendrites were counted it is possible that some dendritic processes were counted as cells, resulting in an overestimation of the numbers of Langerhans cells present in the skin. It is however possible that in our model significant LC migration did not occur. To our knowledge there are no previous *in vivo* human models of secondary immune memory responses where responses of LCs to intradermal injection of antigen has been investigated. It is of interest that in Langerhans cell knock out mice there are enhanced DTH responses[361], suggesting that LCs are not necessary for DTH responses in mice. In addition, a recent study has shown that in psoriasis, a chronic inflammatory skin condition, there is reduced LC migration[362]. The authors proposed that impaired migration may lead to enhanced responses due to localized antigen presentation in the skin.

Previous studies on ageing skin have identified a reduction in the absolute numbers of Langerhans cells within both photoprotected and photoaged non-inflammed skin. We did not identify any changes in the numbers of Langerhans cells in normal skin in the young and old individuals that were used in this study. However, epidermal thinning was observed in the old group, with smaller overall area of epidermis. Thus, there may have been a proportional reduction in LC numbers that we did not detect. It is of interest, however that several studies have reported no changes in the numbers of LCs within the skin in the old and certain mouse models have shown an increase in LC numbers in photoprotected skin. Changes in LC morphology have also been identified in the old with reduced dendritic processes[241]. This was not observed in our study.

An increase in the number of CD1a+ cells was seen in this study around blood vessels during the course of an immune response in the skin. This has

also been reported by previous investigators in addition to an increase in the total numbers of CD1a+ cells in the skin. This observation may be representative of migration of DCs into the skin from the peripheral blood during an inflammatory response in order to enable the repopulation of the skin and may also allow for increased antigen presentation and amplification of the immune response. Alternatively, the increased number of CD1a+ cells may be reflective of the induction of CD1a expression on skin resident cells as a result of the local inflammatory milieu. *In vitro*, immature DCs express a variety of chemokine receptors including CCR1[363], the ligands for which are MIP-1α and RANTES. The levels of both of these chemokines are reduced in old skin and may result in the reduced recruitment of immature DCs during course of immune response in the old.

7. Summary and future directions

In this thesis the effects of ageing on cutaneous immunity were investigated. In particular I investigated whether the reported reduction in DTH responses in the skin were as a result of a previously assumed overall decline in immune responses in the old or whether changes specific to the skin could account for observed reduction in clinical response to the injection of antigen in to the skin. Using bacterial, fungal and viral antigens it was possible to examine both clinical and cellular cutaneous responses in young and old skin. To perform these studies the complimentary techniques of skin suction blister induction and histological analysis of skin biopsies were used. The use of skin suction blisters enabled the isolation of lymphocytes and monocytes directly *ex vivo* from the site of the immune response that allowed for various phenotypic and functional characteristics to be assessed. In addition, the collection of blister fluid allowed for the quantification of the production of chemical mediators at the site of the response. Histological analysis of skin biopsies allowed for the identification and quantification of cells present at the site of the response and their location within the skin.

Only human models were used in order to study immune responses in the skin. Ethical and practical constraints in humans make *in vivo* investigation of the immune system difficult. However, there are several indications that the use of murine models to investigate cutaneous immunity may not accurately reflect humans. In particular, the differences in anatomical sites utilised for the study of DTH responses, the time course of the response and possible differences in lymphoid tissue involvement during recall responses. Further the use of mice to study ageing is also complicated by the vast difference in life expectancy between mice and humans and important differences in lymphocyte differentiation with ageing. We therefore felt justified in using human models alone for the purpose of this study.

The lack of homogeneity between individuals recuited for studies on ageing adds complexity to human studies. Previous studies investigating the effects

of ageing on cutaneous immune responses have been performed on nursing home residents[160] with significant comorbidities and so the interpretation of this data is difficult in the context of defining change as a result of intrinsic ageing alone. A modified version of the SENIEUR protocol was employed in this work in order to reduce confounding factors due to associated significant comorbidity but without excluding significant numbers of old individuals. The aim of the SENIEUR protocol is to reduce confounding factors by excluding individuals with disease[277;277]. However, it has been predicted that this protocol excludes over 90% of the aged population[278] and is therefore selecting individuals who are poorly representative of the ageing population as a whole. It is important that research can be utilised with a view to future therapeutic benefit. The modified version of the SENIEUR protocol used in this study was aimed at selecting a group of well- characterised healthy old individuals, representative of the ageing population as a whole.

We have made several novel observations. Firstly, we have identified a group of healthy old individuals who fail to develop a clinical response to the injection of antigen in the skin in spite of good peripheral blood responses to the antigen, implying that the reduced cutaneous response may not be reflective of a reduced global immunity to the antigen. This indicated that there may be a skin-specific defect in immunity that occurs with ageing. Secondly, the reduced clinical response in the skin is also accompanied by a reduced lymphocytic response. Importantly, in the old and the young the degree of clinical response was been shown to be proportional to the lymphocytic response at day 3, the peak of the clinical response, in addition to at day 7 when the peak of the lymphocytic response is observed. Additionally, the lymphocytes present at the site of the response in the old have reduced activation and proliferation markers with a reduced percentage of cells specific for the antigen injected. Thirdly a reduction in various pro-inflammatory cytokines and chemokines has been shown at the site of the immune response in the old, possibly indicative of defective initiation of the immune response.

The observation that old individuals fail to develop a clinical response to the injection of antigen in spite of peripheral blood responses has potentially important implications for the use of skin testing. The Mantoux test is used widely to assess for both immunity to and potential infection with tuberculosis. This work suggests that cutaneous immune responses are not reflective of global immunity as a whole and therefore the use of skin testing as a way of assessing overall cell mediated immunity to certain antigens cannot be used in the old. The ability of PBMCs to proliferate *in vitro* in response to antigen was used as a measure of immune responsiveness to antigen in our young and old groups. It is possible that the simplified use of *in vitro* proliferation assays is not truly representative of systemic immune responses as a whole. However, *in vitro* proliferation assays require intact antigen presentation and lymphocytic responses in order for cellular proliferation to occur and this did not appear to be compromised in the old group.

The apparent dissociation between cutaneous and systemic immune responses in the old also suggests that there may be a skin-specific decline in immunity that occurs with ageing. This observation is supported by the observation that various skin cancers increase with age[150] whereas there is an improvement in atopic dermatitis[295], an inflammatory skin disease. As far as we are aware there are no previous reports of an organ specific decline in immunity with ageing, suggesting that the skin may be unique in this aspect. In fact, many authors have reported an increase in inflammation with ageing- so called "inflammaging", that is thought to explain the increase in several age-related diseases including ischaemic heart disease[144].

The data from this work suggests that there may be various defects in immune responses that account for the reduction in cutaneous immunity with ageing. Successful cell mediated immune responses in the skin require initiation with recruitment of cells to the site of infection, localised proliferation of cells and then resolution with either emigration or apoptosis of the cells present at the site. A balance between these processes is crucial in order to mount an adequate immune response but also to prevent the development of

pathology due to inappropriate persistent inflammation. Potential changes in cell-mediated immunity in ageing skin are highlighted in Figure 7.1.

Following the findings from this thesis three potential mechanisms are proposed that may account for the observed reduction in immunity:

1. Defective cellular recruitment/migration into the skin.
2. A defect in skin microenvironment such that immune responses are not supported.
3. Defective initiation of immune responses in the skin.

1. Defective cellular recruitment/migration into the skin.

The finding that there are reduced numbers of lymphocytes present at the site of the response in the skin in the old may indicate that there is defective migration of cells from the peripheral blood into the skin. Reduced numbers of CD4+ T lymphocytes were observed at all of the time points studied in the old, seen in association with a reduced clinical score. We investigated the expression of various cell surface receptors on lymphocytes, derived from peripheral blood in the young and old, thought to be important for cellular recruitment in to the skin. Although no difference in the expression of the key skin homing receptors CLA, CCR4 and CD11a was identified, we have identified a reduction in the expression of CXCR3 on CLA+ T cells in the old compared with the young.

It remains unclear as to whether the observed reduction in CXCR3 expression has an effect on the migratory capacity of cells in to the skin and if there is some redundancy in receptor expression. Very recent work, however, has suggested that whilst CXCR3 expression on T lymphocytes is important for responses to PPD antigen, the expression of CCR4 is important for responses to Candida[364]. Thus, whilst a reduction in CXCR3 expression could explain the observed reduction in responsiveness to PPD antigen in the old, it may not account for the observations seen with Candida antigen. CXCR3 expression is known to be upregulated on T lymphocytes at sites of inflammation[310] and it has been proposed that this may be an important mechanism for cellular retention. Future work to investigate

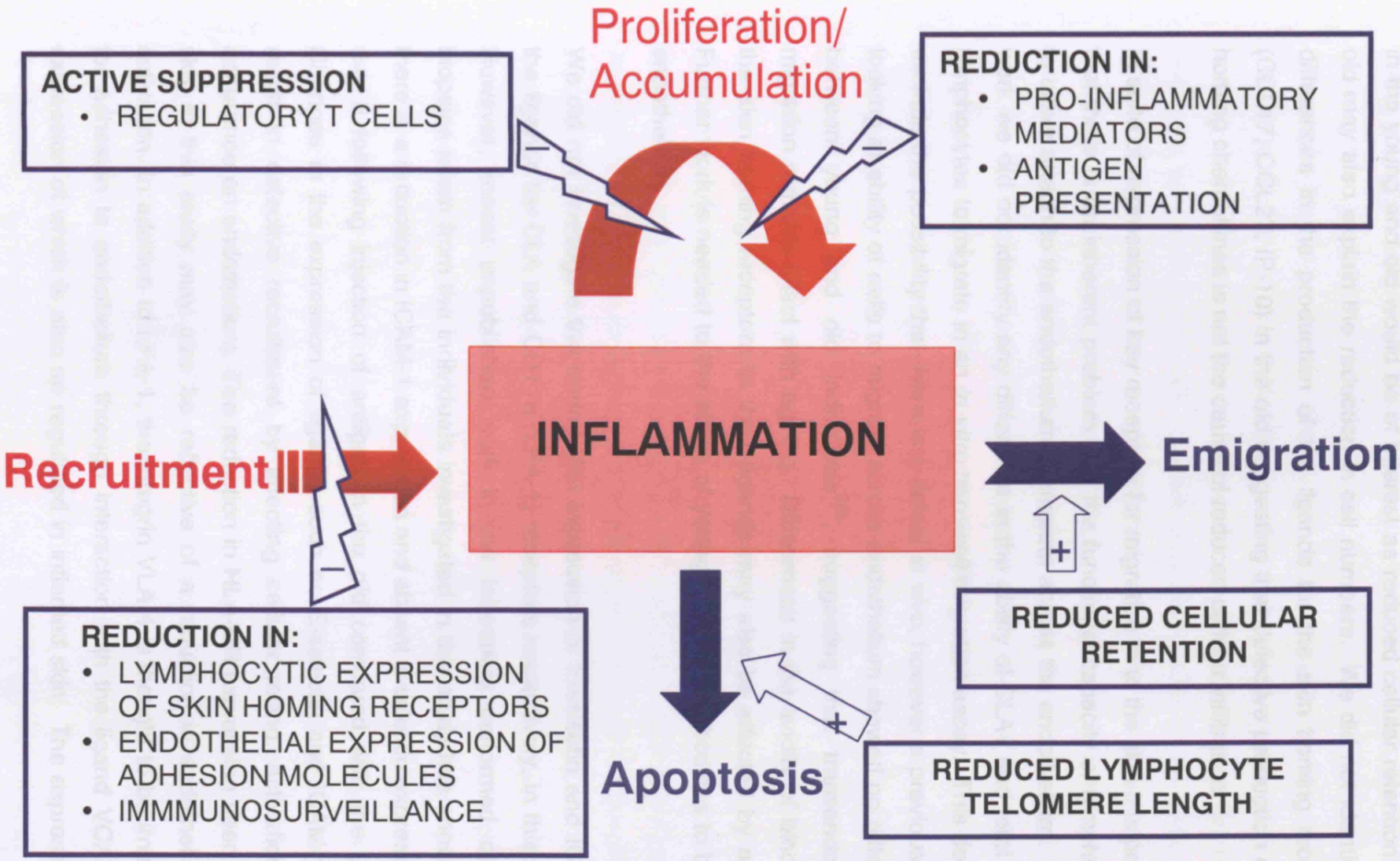


FIGURE 7.1

MECHANISMS FOR REDUCED CUTANEOUS IMMUNITY IN THE OLD

CXCR3 expression on T lymphocytes within the skin at sites of inflammation in the young and old would be of interest as reduced cellular retention in the old may also explain the reduction in cell numbers. We did not identify any differences in the production of the ligands for the skin homing receptors (CCL17, CCL27, IP-10) in the old suggesting that defective production of skin homing chemokines is not the cause of reduced cellular infiltration.

In spite of expression of key receptors for migration in to the skin it is possible that there is an inherent problem with the functional capacity of lymphocytes to either attach to the endothelium or migrate across the endothelium. In this work we did not identify any difference in the ability of CLA+ and total CD4+ lymphocytes to migrate in an *in vitro* transwell migration assay. This does not exclude the possibility that there is a defect *in vivo*, however a previous study looking at ability of cells to migrate across endothelium showed no difference between young and old individuals[365], suggesting that transendothelial migration remains intact with ageing. Differences in the avidity of binding of the skin homing receptors to their ligands may also be affected by ageing. Further work is needed to the ability of young and old lymphocytes to bind to endothelium.

We did not investigate the endothelial expression of E-selectin and ICAM-1, the ligands for CLA and CD11a (LFA-1) receptors respectively, in this study. However, recent unpublished work in our laboratory performed on skin biopsies taken from the individuals investigated in this study has shown that there is a reduction in ICAM-1 expression and absent E-selectin expression at day 3 following injection of antigen in the old compared with the young. Changes in the expression of ligands such as E-selectin and ICAM-1 may result in defective recruitment by affecting cellular rolling, activation and adherence on endothelium. The reduction in HLA-DR expression seen in old skin in this study may also be reflective of a reduction in endothelial cell activation. In addition to LFA-1, the integrin VLA-4 is thought to be important for adhesion to endothelium through interaction with the ligand VCAM the expression of which is also up regulated in inflamed skin. The expression of

VLA-4 on T lymphocytes in the peripheral blood was not investigated in this study.

There is limited evidence from previous studies to suggest that the expression of E-selectin, ICAM-1 and VCAM is altered in ageing skin. A previous study of wound healing in humans has indicated that there is an altered inflammatory response and endothelial cell adhesion molecule profile in old skin with a delay in lymphocyte and monocyte infiltration in to the skin associated with increased early expression of E-selectin but delayed endothelial expression of ICAM-1 and VCAM-1[366]. Altered expression of ICAM-1 in response to IFN-γ has also been seen in basal cell carcinomas[367], a common form of cutaneous neoplasia in the old. In future work it would be of interest to investigate whether similar changes were present in an antigen-driven model of inflammation in old skin.

Changes in the blood supply to the skin could also account for a reduction in cellular recruitment seen in the old. Certain changes to the cutaneous vasculature are well documented in old skin[250;257;258] and are outlined in the introduction, although marked variability in vasculature has been reported depending on the site investigated and the degree of photo-exposure[260]. In this study, although there was an overall reduction in the numbers of lymphocytes seen within perivascular infiltrates, the actual number of perivascular infiltrates was not significantly different between the young and old groups. This indicates that there was not a marked reduction in the actual number of dermal blood vessels in the old individuals studied. The degree of erythema and induration seen as part of the clinical response at the test site is dependent on both blood flow and vasodilatation. Changes in these parameters could potentially explain the reduced or absent clinical response in the old and in future it would be of interest to assess this using laser Doppler technology, previously employed to assess the Mantoux test response in the skin in young individuals[253].

It is possible that there is defective immunosurveillance in old skin. The trafficking of lymphocytes through the skin may not only be important for detecting potential pathogens but may also have an important role in preventing skin neoplasias. The CCL1/ CCR8 axis has been demonstrated to be important for cutaneous immunosurveillance[66]. We did not detect any difference in the levels of CCL1 in blister fluid from blisters induced over either normal skin, following saline injection or after Candin injection, suggesting that CCL1 may not be involved in acute inflammatory responses. A possible reduction in CCR8 expression on CLA+ peripheral blood T lymphocytes was found although the population of cells analysed was too small population to draw definite conclusions. As the majority of CLA+CCR8+ lymphocytes are found in the skin it would be useful to analyse this population in young and old either by staining skin biopsies or analysing cells from skin suction blisters.

2. Defect in the skin microenvironment.

In spite of the apparent reduction in the numbers of CD4+ lymphocytes present at the site of the immune response in the skin a significant reduction in other cell types including CD8+ lymphocytes and monocytes was not seen. Although it is possible that there there is a CD4+ T lymphocyte- specific defect in migration in to the skin, the finding of similar numbers of other cell types suggests that changes in cellular recruitment into the skin alone cannot completely explain the observed reduction in immune responses in the old. One possible explanation is that the local microenvironment of the skin is unable to support or maintain immune responses once cells have migrated to the site of the response.

Our laboratory has previously shown that during the course of the Mantoux Test response the expansion of CD4+ T lymphocytes is dependent on the localised proliferation of cells within the skin rather than ongoing recruitment from the peripheral blood[88]. This conclusion is supported by several observations; that the cells in the skin express high levels of the proliferation marker Ki67, that they bear the same clonality of T cell receptor and that no detectable changes in cellular proliferation or activation occur in the peripheral blood during the course of the immune response. It is therefore possible that

although there is initial recruitment of CD4+ lymphocytes into the skin in the old these cells are unable to become activated and proliferate locally resulting in reduced expansion of the CD4+ lymphocyte population.

Using the skin suction blister technique, we have demonstrated that CD4+ lymphocytes examined directly *ex vivo* from the site of the immune response in the old have reduced expression of CD69, suggesting reduced cellular activation, and Ki67, indicating that fewer cells are proliferating at the site. In particular, the finding that there are reduced percentages of antigen specific T lymphocytes in the old may reflect the inability of this cell population to expand within the skin in the old.

At present, little is known about the effects of ageing on cytokine/ chemokine production in humans and studies on their production have produced varying and conflicting results. This may be reflective of the different study populations selected and the nature of the assays used to assess chemokine and cytokine production. *In vivo* studies in mice and humans have investigated the results of intradermal injection of different chemokines and cytokines in to the skin, however the cutaneous response to infection is likely to activate production of numerous chemokines making the interpretation of these studies difficult. The use of skin suction blister fluid analysis in this study allowed for the quantification of multiple chemokines and cytokines at the site of an evolving immune response.

We have identified reduced concentrations of various pro-inflammatory mediators in the blister fluid from blisters induced over the site of immune responses to candida in the old. The reduction in concentrations of TNF-α and IFN-γ could be explained by the lack of T lymphocyte numbers and activation in that both are produced by activated T lymphocytes. Both of these mediators also have an important role to play in the amplification of the immune response. TNF not only acts to increase blood flow and vascular permeability but also to increase the recruitment of T lymphocytes to the site of inflammation by increasing the expression of E-selectin, ICAM-1 and

VCAM-1 on the endothelial cell surface[101]. Thus a reduction of TNF production in the skin could explain the observed clinical response and reduced number of lymphocytes in the old. However, a reduction in TNF levels is unlikely to be the sole reason for reduced responses in the old in that in the few old individuals who did have a good clinical and cellular response to the injection of antigen low concentrations of TNF were measured in the blister fluid.

It is also possible that there is active suppression of immune responses in the skin in old individuals. Recent interest in a subgroup of T lymphocytes with ability to suppress the immune response known as suppressor or regulatory T cells. Of interest this group of cells expressing CD25 receptor are enriched in skin homing receptors including CLA, CCR4 and CCR8[368] while they are relatively depleted in gut homing receptor expression[369;370]. CD4+CD25+ suppressor cells isolated from cord blood are deficient in CCR4 expression and it is therefore possible that CCR4+CD25+CLA+Ts cells may be generated in the periphery after birth in response to skin-associated antigens[371]. The peripheral generation of T regs is also supported by previous work from our laboratory[371]. An increase in the number of circulating peripheral regulatory T cells has been reported in the old[372]. It is therefore possible that, with ageing, there is a progressive accumulation of regulatory T cells with suppressive properties that are specific for cutaneous antigens that result in diminished responses in the skin. Future work studying the role of these cells during acute inflammatory responses in both the young and old would be of interest. Other potential mechanisms of active suppression such as IL-10 production and expression of the inhibitory receptor ILT3 on monocytes were investigated but did not show any differences between the young and old groups.

Although we have not detected a reduction in the functional capacity of T lymphocytes isolated from peripheral blood in the old it is possible that the function of lymphocytes recruited into the skin is impaired. A process known as replicative senescence, where there is loss of T cell telomere length due to cellular replication with subsequent apoptosis, is thought to prevent over-expansion and inappropriate inflammation in normal skin. This process is

unlikely to place restraints on the development of immune responses in the young. However, in the old it is known that both CD4 and CD8 subsets of T cells have significantly shorter telomere lengths[204] and therefore cutaneous T cell telomere shortening may result in diminished immune responsiveness due to limited replicative potential of T cells. Although, to a certain extent telomere length can be restored by the cellular induction of telomerase, previous work from our group has shown that the production of IFN-α within the skin at the site of an immune response has an inhibitory effect on this enzyme[88]. Ageing cells may also have a reduced capacity to induce telomerase[202], placing further limitations on the replicative potential of ageing T lymphocytes. Future work to study the effects of ageing on telomere erosion, telomerase inducibility and IFN-α production during an acute immune response in the skin would help to clarify whether the reported reduction in telomere length in ageing lymphocytes has an effect on cell-mediated responses in the skin.

It may be possible to induce telomerase in ageing cells[196]. Gene therapy studies on cells in culture have demonstrated that the process of replicative senescence can be retarded by the manipulation of telomerase. Gene transduction with the catalytic component of human telomerase (hTERT) was performed on a variety of skin-derived cells including human fibroblasts, epithelial cells and keratinocytes[201]. Introduction of hTERT led to unlimited proliferation, telomere length stabilisation and normalisation of function of the cell populations studied. Similar results found for HIV-specific CD8+ cells from individuals with HIV. This type of treatment has been proposed as potential therapeutic target not only for HIV but also as a means of enhancing viral immunity and responses to vaccines in the old[201].

3. Defect in the initiation of the immune response.

Innate immune responses are critical for the initiation of immune responses in the skin and are required for effective adaptive immune responses to develop[373]. Antigenic infection of the skin is thought initially to result in the activation of resident innate immune cells such as Langerhans cells, dermal dendritic cells and monocytes through various mechanisms including Toll-like

receptors, surface bound immunoglobulin and exposure to pro-inflammatory cytokines such as IL-1 and TNF-α released by keratinocytes and dendritic cells in response to epithelial trauma.

We were unable to detect any significant differences between the young and old in the numbers of antigen presenting cells in the skin as determined by CD1a and DC-SIGN expression. However, a possible reduction in HLA-DR expression was seen indicating that antigen presenting function in the skin in the old may have been compromised with reduced activation of dendritic cells. There is currently conflicting evidence regarding the effects of ageing on antigen presention by dendritic cells, with reports of both reduced and enhanced antigen presenting capacity in the old[232;235]. Future work using the skin suction blister technique to extract antigen-presenting cells from the site of a dynamic immune response may permit a better understanding of the antigen presenting capacity of cells within the skin in the old.

Plasmacytoid dendritic cells (PDCs), derived from lymphoid progenitors, may also have an important role in antigen presentation in the skin. They are present in very small numbers in normal skin, however large numbers of PDCs have been found to be present in allergic contact dermatitis[374], psoriasis[375] and cutaneous lupus erythematosis[376]. The role of PDCs in DTH responses is unknown and is currently under investigation in our laboratory. PDCs produce IFN-α, known not only to inhibit telomerase within the skin[88] but also to have an important role in anti-viral responses. In particular, IFN-α has been shown to induce virus specific CD4+ cells within the peripheral blood to express the skin homing marker CLA[377].

Monocytes play an important role in the skin in both local antigen presentation in addition to the production of pro-inflammatory mediators. The reduction in levels of TNF, MIP- 1α, MCP-1 and RANTES raises the possibility that there is a defect in monocytes in ageing skin in that all of these cytokines/ chemokines are produced by monocytes and are also responsible for monocyte activation. Defective production of monocyte- derived chemokines

may also impact on lymphocyte recruitment in to the skin as a previous study in humans has shown that the injection of MIP-1α results in upregulation of E-selectin on endothelium[331]. We were unable to identify any significant change in either the numbers of monocytes in skin or their activation in the old. However only small numbers of old individuals were studied, some of whom had good immune responses in the skin, and so the individuals recruited in this study may not have been representative of the old population as a whole. It is also possible that in spite of the expression of normal levels of monocyte activation markers on skin-derived monocytes in our study there was a defect in the ability of these cells to produce chemokines. This requires further investigation.

In addition to acute inflammatory responses to pathogens, macrophages are also thought to have an important role in wound healing. It is well recognised that wound healing in the old is impaired which results in considerable morbidity and also financial costs in healthcare provision[378]. MCP-1 has been found to be important in both mice and humans for effective wound healing by influencing macrophage effector state[227;349;379]. Defective macrophage function may therefore be responsible for deterioration in wound healing with ageing. Identification of the mechanism for the reduced production of monocyte- derived chemokines, as found in our study, may therefore help to provide therapeutic targets for improving not only cutaneous immunity but also wound healing in the old.

Although there is very limited information on the effects of ageing on toll-like receptor (TLR) expression in humans, murine studies have indicated that the expression of some toll- like receptors (TLRs) is reduced with ageing[221]. The use of imidazoquinolones, such as Imiquimod, for the treatment of various cutaneous diseases including basal cell carcinomas and genital warts is currently increasing. Importantly, Imiquimod is used commonly in the old for the treatment of basal cell carcinomas and is thought to result in tumour clearance by inducing an inflammatory response in the skin[380]. Imiquimod is thought to work by stimulating the innate immune system via TLR7 and has

been shown to induce the production of IFN-α[380], TNF-α[381], MIP-1α and MCP-1[380]. It has also been shown to induce IFN-γ production in T cells either directly or via T-cell receptor triggering[382]. It is therefore possible that a defect in cutaneous immunity in the old, resulting in diseases such as basal cell carcinoma, may be overcome by pharmaceutical methods. Future studies investigating whether the application of Imiquimod can overcome poor responsiveness to antigen in old using the models used in this thesis may provide clues regarding changes in innate immunity in ageing skin.

In conclusion, both reduced clinical and lymphocytic responses to antigen in old skin have been identified. The observed reduction in immunity in ageing skin may be multifactorial. However, a defect in the initiation of the response by the innate immune system would explain the reduction in the production of pro-inflammatory mediators, cellular activation and endothelial expression of ligands with a resulting reduction in lymphocyte recruitment and proliferation. The identification of a mechanism for the apparent skin-specific reduction in immunity seen in the old may in the future be able to identify therapeutic targets in order to selectively enhance cutaneous immunity.

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